DEGRADATION AND AQUATIC TOXICITY OF OIL SANDS NAPHTHENIC ACIDS USING SIMULATED WETLANDS

A Thesis Submitted to the College
of Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Toxicology Graduate Program
University of Saskatchewan
Saskatoon, Saskatchewan
Canada

By
Navdeep Singh Toor
Winter 2012

PERMISSION TO USE

In presenting this thesis/dissertation in partial fulfilment of the requirements for a Doctor of Philosophy degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis/dissertation in any manner, in whole in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis/dissertation work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis/dissertation.

DISCLAIMER

Reference in this thesis/dissertation to any specific commercial products, process, or service by trade name, trademark, manufacturer, or otherwise, does not constitute or imply its endorsement, recommendation or favouring by the University of Saskatchewan. The views and opinions of the author expressed herein do not state or reflect those of the University of Saskatchewan, and shall not be used for advertising or product endorsement purposes.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Chair of the Toxicology Graduate Program
Toxicology Centre
University of Saskatchewan
44 Campus Drive
Saskatoon, SK, Canada S7N 5B3

ABSTRACT

Oil sands process-affected waters (OSPW) from the Athabasca oil sands (AOS) located in northern Alberta, Canada, are toxic to aquatic organisms due to the presence of organic and inorganic constituents. Much of this toxicity is related to a group of dissolved organic acids known as naphthenic acids (NAs). Naphthenic acids are a natural component of bitumen and are released into process water during the caustic hot water extraction process used to separate the bitumen from the oil sand ore. This complex mixture of non-cyclic and mono- and poly-cyclic alkanes containing carboxyl groups are characterized by the general formula $C_nH_{2n+z}O_2$, where n indicates the carbon number, and Z represents the number of fused rings in the structure. Currently, all process-affected waters are stored within large holding ponds and settling basins on the oil sands mining lease sites with the understanding that eventual reclamation of this water must be undertaken. Successful reclamation of OSPW is expected to require a reduction in total NAs concentrations in the OSPW and the removal of the toxic character of the water. Natural or enhanced bioremediation in lakes and wetlands within the lease closure landscapes will play a critical role in meeting these two requirements. This research investigated the potential for the reduction of total NAs concentrations in OSPW due to biotic (e.g., biodegradation) and abiotic (e.g., sorption) processes, and its relationship to the overall toxicity of OSPW. The specific goals of this research were to determine if natural degradation of NAs in simulated wetland environments could be enhanced by manipulating various physical and chemical factors of the environment, to describe and quantify the selective biodegradation rates of NAs congeners, and to correlate observed changes in total NAs concentration and composition with changes in the aquatic toxicity of OSPW.

The complexity of both OSPW and NAs mixtures presented an unusual set of challenges. A preliminary investigation was used to determine the contributions of salinity and NAs to the total aquatic toxicity of OSPW in order to identify a suitable test organism that would respond to NAs concentrations while tolerating the high ionic content of OSPW for the main simulated wetland microcosm study. Seven-day *Ceriodaphnia dubia* chronic toxicity tests, using both un-manipulated (containing NAs) and manipulated (substantially reduced NAs) samples of OSPW, identified salinity as a potential contributing factor to the overall toxicity of this complex water.

Only a 5% reduction in acute toxicity and an 11% reduction in chronic toxicity was observed with a 91% reduction in total NAs concentration (from 67.2 to 5.9 mg/L; removed by solvent extraction). However, when the same samples were tested using the salt tolerant bacteria *Vibrio fischeri* in the Microtox® bioassay system, the 91% reduction in total NAs concentration, the toxicity was removed (EC_{50} changed from 57.8 to >100%). These results suggested that salts in OSPW may drive the toxicity of OSPW to some freshwater invertebrates, such as *C. dubia*, and that the Microtox® bioassay was better suited to track the overall toxic potential of NAs in OSPW.

Using flow-through, laboratory microcosms to mimic natural wetlands, it was demonstrated that the reduction in total NAs concentration, based on the Fourier Transform Infrared (FTIR) spectroscopy analysis, was dependent upon hydraulic retention time (HRT), but appeared to be unaffected by nutrient addition (nitrogen and phosphorus). Microcosms with a longer HRT (for two OSPW types; Syncrude and Suncor) showed higher reductions in total NAs concentrations (64 to 74% NAs reduction) after the 52-week test period, while nutrient enrichment appeared to have little effect. While the total NAs concentrations decreased in the waters from the microcosms, a 96-hr static acute rainbow trout (*Oncorhynchus mykiss*) bioassay showed that the initial acute toxicity of Syncrude OSPW (LC_{50} = 67% v/v) was reduced (LC_{50} >100% v/v) independent of HRT. However, EC_{20}s from the Microtox® bioassays were relatively unchanged when comparing the input and output microcosm waters maintained at both HRTs over the 52-week study period, indicating that some sub-lethal toxicity persisted under these experimental conditions. The study demonstrated that given sufficiently long HRTs, simulated wetland microcosms containing OSPW significantly reduced total NAs concentrations and acute toxicity, but left behind a persistent component of the NAs mixture associated with residual toxicity.

Further investigations aimed to describe and quantify the selective biodegradation of NAs congeners and correlate the observed changes in total NAs concentration and composition (i.e., NAs fingerprint profile) with the aquatic toxicity of OSPW. High performance liquid chromatography/quadrupole time of flight-mass spectrometry (HPLC/QTOF-MS) analysis was used to track the changes in NAs mixture profiles or 'fingerprints' in each experimental treatment over time. Based on first-order degradation kinetics, rapid degradation was observed

for NAs that had lower carbon numbers (11 to 16) and fewer degrees of cyclization (*Z* series -2 to -4; half-lives between 19 to 28 weeks). Within the NAs mixture fingerprint, the two most persistent groups of NAs homologues were identified (NAs with carbon numbers 17 to 20 and *Z* series -6 to -12; half-lives between 37 to 52 weeks). Their persistence may have resulted in the residual chronic toxicological response as measured by the Microtox® bioassay (EC_{20}).

An additional study was conducted to characterize potential changes in the total concentration and composition of NAs in OSPW due to sorption to organic wetland sediments. The batch-reactor investigation showed a rapid (<1 day) and significant reduction in total NAs concentrations in OSPW when mixed with the wetland sediment at a ratio of 2:1 v/v (OSPW:sediment). The mean percent reduction of NAs in OSPW was 67% during the 14-day test period, suggesting a significant influence of sorption on the removal of NAs than previously expected. However, no preferential sorption was observed based on the distribution of NAs congeners with respect to carbon number, *Z* series, and arbitrarily defined clusters. The potential sorption of OSPW NAs as a result of using substrates with high organic carbon content (e.g., 27.6% total organic carbon content) in designed wetlands may enhance the mitigative capabilities of these reclamation landscapes at the AOS. Further investigations into understanding NAs sorption kinetics without substrate agitation are warranted before these results can be extrapolated to the field.

Finally, to test the hypothesis that persistent components of an OSPW NAs mixture (e.g., NAs congeners with higher carbon numbers and degrees of cyclization) may be responsible for the observed residual chronic toxicity identified in the previous simulated wetland microcosm study, the fractionation of OSPW NAs was attempted using both off-line anion exchange chromatography and batch-wise co-polymer filtration and elution. Although complete separation was not achieved in this investigation, the results suggested that specific variations of the co-polymer were most effective and showed the most promise for separating the NAs mixtures based on polarity and size. With further refinements to the procedure, future investigations may be able to achieve adequate separation of the NAs mixture into fractions with compositions different enough to conduct toxicity bioassays.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor, Dr. Karsten Liber, for the opportunity to pursue this research. Thank you for your guidance and wisdom, and for providing me the skills and confidence to succeed as a scientist.

I would also like to thank the members of my advisory committee, Drs. Barry Blakley, John Headley, Paul Jones, Steven Siciliano, and Lee Wilson for their contributions and support. I would like to acknowledge the assistance of Dr. Mike MacKinnon formerly of Syncrude Canada Ltd. for his valuable insight into the oil sands industry and for providing some of the analytical support conducted as a part of this thesis. Funding for this research was generously provided by the Cumulative Environmental Management Association (CEMA), Canadian Oil Sands Network for Research and Development (CONRAD), the NSERC-CRD-funded Carbon dynamics, food web structure and reclamation strategies in Athabasca oil sands wetlands (CFRAW) project, and the Toxicology Graduate Program, University of Saskatchewan. I could not have completed this research without the support of faculty, technicians, administrative staff (Adriana, Fiona, and Shanda), friends, and fellow graduate students at the Toxicology Centre.

To my extended family in Saskatoon, Chris Ryan (Saucy), Matt Anderson (Bo Duke), Steve Seiferling (Scuba Steve), Clark Robertson (Pig Dog), Jeremy Cardinal-Schultz (Bones) and the whirlwind of people that surround them at any given moment, thank you for not letting me forget what's important, and for the juice whenever my batteries needed recharging. I would like to thank the Gophers Rugby Club in Saskatoon - instantaneously, twenty-two of the best friends a guy could have when moving to a new city. I wish to thank all my friends originally from Kitimat, BC, with whom I share an unconditional bond that never requires validation. I would like to thank Kendra Munday for her support during the last big push of writing this thesis.

And finally, I would like to thank my family for their patience and encouragement. Especially my parents, Manjit and Surinder, who have always given me the freedom to choose without questions or conditions. My younger brother Mick, who never fails to put things into perspective and is always there when you need him. My sister and brother-in-law, Sonia and Gary Mehat,

who always make my visits exciting and extremely memorable. Thank you for being my home-base and contact to everything I miss while away. And last but not least, my favourite nephews and niece, Aris, Tyas, and Mila Belle, who I wish I could spend more time with and are my inspiration in all things life. The future of this planet is in great hands.

This thesis is dedicated to family and friends who passed away during the course of my studies and were not able to see or celebrate its completion.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AEO	acid-extractable organics
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AOS	Athabasca oil sands
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
BDL	below detection limit
C.I.	confidence interval
CFMW	carbon-filterd municipal water
CMC	critical micelle concentration
CNRL	Canadian Natural Resources Limited
CT	consolidated tailings
CV	compensation voltage
DCM	dichloromethane
DO	dissolved oxygen
DOC	dissolved organic carbon
EC20	effective concentration (20%)
EC50	effective concentration (median, 50%)
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EI	electron impact
EPEA	Environmental Protection and Enhancement Act
EROD	ethoxyresorufin-*o*-deethylase
ESI	electrospray ionization
FABMS	fast atom bombardment mass spectrometry
FAIMS	high-field asymmetric wave-form ion mobility spectrometry
FID	flame ionization detection
FTICR	Fourier transform ion cyclotron resonance mass spectrometry
FTIR	Fourier transform infrared
GC	gas chromatography
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
HRT	hydraulic retention time
IC20	inhibition concentration (20%)
IC50	inhibition concentration (median, 50%)
ICP-OES	inductively coupled plasma optical emission spectrometry

JACOS	Japan Canada Oil Sands
LC100	lethal concentration (absolute, 100%)
LC50	lethal concentration (median, 50%)
LD50	lethal dose (median, 50%)
LRMS	low resolution mass spectrometry
LSIMS	liquid secondary-ion mass spectrometry
m/z	mass to charge ratio
MIBC	methyl isobutyl carbinol
MLSB	Mildred Lake Settling Basin
MS	mass spectrometry
MTBSTFA	N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide
NAs	Naphthenic acids
NOEC	no observed effects concentration
NPH	2-nitrophenylhydrazine
OSPW	oil sands process-affected waters
OSWRTWG	Oil Sands Water Release Technical Working Group
PAH	polycyclic aromatic hydrocarbons
QTOF	quadrupole time of flight
Recon.	reconstituted
SD	standard deviation
SE	standard error
SPE	solid phase extraction
SSB	Syncrude Sweet Blend
***t*-BDMS**	the *tert.*-butyldimethylsilyl
T3	triiodothyronine
T4	thyroxine
TAN	total acid number
TEA	triethanolamine
TEH	total extractable hydrocarbons
TOC	total organic carbon
TSK	trimmed Spearman-Karber
YCT	yeast, Cerophyll™, and trout chow

PREFACE

This thesis has been organized as series of manuscripts for publication in scientific journals. Some repetition of introductory and methodological material is therefore unavoidable. References cited in each chapter have been collated in the reference section at the end of this thesis (Chapter 8). References cited in the appendices are listed separately in the Reference List For Appendices section.

1.0 GENERAL INTRODUCTION

The extraction and processing of oil sands has resulted in a number of environmental issues at the Athabasca Oil Sands (AOS) including the generation of enormous volumes of liquid tailings waste and oil sands process-affected waters (OSPW). Syncrude Canada Ltd. (Syncrude) and Suncor Energy Inc. (Suncor) are two of the growing number of Canadian and international companies currently involved in oil sands development at the AOS. Both companies contribute to the accumulation of substantial volumes of OSPW.

Although OSPW can contain a complex mixture of organic and inorganic constituents, the acute and chronic aquatic toxicity has been largely attributed to a relatively persistent group of dissolved organic acids known as naphthenic acids (NAs) (MacKinnon and Boerger, 1986; Verbeek, 1994; Headley and McMartin, 2004; Quagraine et al., 2005b). Naphthenic acids are a natural component of petroleum, and may naturally be released into the aquatic environment in those rare instances where petroleum deposits come in contact with surface water bodies and groundwater aquifers (Schramm et al., 2000). Background surface water NAs concentrations in the AOS region have been detected at concentrations below 1 to 2 mg/L (FTFC, 1995b; CEATAG, 1998; Leung et al., 2003) and concentrations of NAs in near-surface groundwater aquifers have been measured in the range of < 2 to 20 mg/L (CEATAG, 1998). However, as a result of the hot water extraction process, NAs become increasingly soluble and become concentrated in process and tailings waters as sodium naphthenates. Previous studies have measured NAs in AOS OSPW at concentrations ranging from 20 to 120 mg/L (Leung et al., 2001; Clemente and Fedorak, 2005).

Naphthenic acids are a complex mixture of non-cyclic and mono- and poly-cyclic alkanes that contain carboxylated aliphatic side chains of various lengths. They are commonly characterized by the general chemical formula $C_nH_{2n+z}O_2$, where n indicates the carbon number, and Z represents the homologous group series number (i.e., the number of hydrogen atoms that are lost as the structures become more compact or as the fused-ring number increases). Moreover, cyclic NAs are believed to be substituted with alkyl groups, usually consisting of a small methyl or ethyl group, denoted by the letter *R*. For example, the Z value is equal to 0 for saturated hydrocarbon chains, and becomes -2 in mono-cyclic NAs, -4 in bi-cyclic NAs, and -6 in tri-

cyclic NAs (Lai et al., 1996; CEATAG, 1998; Schramm et al., 2000; Scott et al., 2005). These highly complex mixtures of carboxylic acids are chemically stable and have dissociation constants between 10^{-5} and 10^{-6} (pK_a between 5 and 6). The solubility of NAs is greatly dependent on the ambient pH of OSPW, which is typically between 8 and 9. Under these alkaline conditions, the non-volatile NAs, having a relatively low fugacity, adsorption coefficient (K_d), and log octanol-water coefficient (log K_{ow}), persist in the water column in the highly soluble, dissociated form (Schramm et al., 2000; McMartin, 2003; Rogers, 2003; Clemente and Fedorak, 2005). The NAs in OSPW consist of hundreds of individual compounds having a wide range of molecular weights. Even within a specific group of carbon number and *Z* series, there can be numerous isomers each having a similar set of physical and chemical properties, making them difficult to analyze and study (Holowenko et al., 2002).

Naphthenic acids are not expected to persist in tailings ponds over long periods of time due to the presence of indigenous microbial communities capable of degrading these organic acids (Foght et al., 1985; Herman et al., 1994b; Del Rio et al., 2006). Previous studies have shown that on-site, aerobic, aquatic reclamation environments without "fresh" OSPW recharge are capable of reducing the concentration of lower molecular weight NAs over time (10 to 15 years). However, this process is slower and less complete with heavier molecular weight NAs (Nix and Martin, 1992; Lai et al., 1996; Quagraine et al., 2005a). Studies such as Holowenko et al. (2002) and Leung et al. (2001) have indicated that biodegradation of NAs is a function of both abiotic and biotic processes, which include the presence of specific types of microbial communities, pond hydraulic retention time (HRT), and nutrient availability.

As a result of the "zero discharge policy", OSPW, containing NAs and other organic and inorganic substances, have been stored on-site and will likely require some level of treatment prior to decommissioning or its discharge into the natural environment. The toxicity of OSPW is, at least partially, related to the NAs. The connection between the initial degradation of specific NAs components and the loss of aquatic toxicity, along with the rate of change, has not been investigated thus far. The experiments conducted in this research address the role and effectiveness wetlands can play in reducing the total concentrations of NAs, identifies and quantifies any selective degradation of NAs congeners, and describes the associated reduction in

acute and chronic aquatic toxicity of OSPW. It is hoped that the results of this research will help guide design criteria for in-situ NAs degradation and wetland reclamation success. It is believed that through improved insight into mechanisms, pathways and rates of detoxification of OSPW, stakeholder confidence in wetlands performance goals within reclamation plans will be increased.

1.1 Oil Sands and Bitumen

Fuels derived from petroleum (*synonym. crude oil*) supply more than half of the world's total energy supply and is perhaps the most important substance consumed in society today (Speight, 1999). The extra heavy oil, a subclass of petroleum referred to as bitumen, has a higher density and viscosity than both conventional and heavy oils and can be found in oil sands deposits often in the same geographical areas as conventional petroleum (Speight, 2000). These oil sands deposits are located all over the world, but the largest are located in Canada (province of Alberta) and the Orinoco region of east central Venezuela, followed by deposits located in northwestern China. Smaller oil sands deposits occur in the United States (mainly in the state of Utah), Peru, Trinidad, Madagascar, the former Soviet Union, Balkan States, and the Philippines (Speight, 1999).

As major conventional fossil fuel supplies are continually depleted on a worldwide scale, oil sands are becoming more important as an alternate but viable fuel source due to its global distribution and available technology to upgrade the bitumen into lighter, more useable liquid products. It has been estimated that there are in excess of 3 trillion (3×10^{12}) barrels or 477×10^9 m^3 of hydrocarbon liquids available from potential reserves occurring in oil sands deposits worldwide (Speight, 2000), although its complete recovery in many locations is limited by economic and technical complications.

Oil sands are a type of petroleum deposit containing sand impregnated with the naturally occurring, dense, viscous bitumen, which may or may not be associated with water. This association of water or lack thereof greatly influences the recoverability of bitumen during extraction operations based on aqueous processes (Takamura, 1982; Rogers, 2003). Bitumen itself is a mixture of saturated and unsaturated hydrocarbons, organometallic compounds,

asphaltenes, and polar constituents (Chilingarian and Yen, 1978). The amount of bitumen present in the oil sands can vary from 0 to 18% on a per weight basis, even within a particular deposit (Speight, 2000). This extra heavy, carbon disulfide-soluble oil can vary in density, viscosity, and chemical make-up depending on the origins of deposition, geological settings, and in situ decomposition (Chilingarian and Yen, 1978; AOSTRA, 1989). As a result, the exact and specific compositional properties are difficult to discern and are most consistent within a given geographical location.

1.2 Athabasca Oil Sands Development and Operations

The AOS is located in the northeast part of the province of Alberta, near Fort McMurray (56°43' N, 111°22' W) (Barton and Wallace, 1979). This area represents one of the world's largest single petroleum deposits where the petroleum exists in the form of bitumen, containing an estimated 200 billion m^3 of bitumen or 1334 billion barrels of oil (Chilingarian and Yen, 1978). Other deposits in the area include Wabasca, Peace River and Cold Lake. The AOS is the largest of the four deposits, covers about 42,000 km^2, and is currently undergoing large-scale commercial exploitation (Gentes et al., 2007a). A previous estimate of the percentage of Canada's synthetic crude oil supply that is mined from the AOS deposit was reported to be between 15 to 20% (Siwik et al., 2000). With the continual growth of the oil sands industry, newer estimates are considered to be greater than 25% (McMartin, 2003), and rapidly increasing to as much as 50% in a few years (Leung et al., 2003). Scott et al. (2008) estimated that in 2005 bitumen from the oil sands accounted for 58 and 39% of total petroleum production in Alberta and Canada, respectively.

Bitumen can be recovered from the subsurface by both mining and non-mining (*in situ*) techniques depending on the properties (composition and formation) of the bitumen reservoir. *In situ* mining techniques involve the injection of a fluid into the oil sand formation, the subsequent displacement of bitumen, and then surface recovery via production wells. Conversely, the surface, open-pit mining method used by most of the original mining companies, to extract bitumen at the AOS, involves mining and transportation of the oil sands ore to a processing plant where the bitumen is extracted and the sand is disposed of as waste (Speight, 2000). This

method is applicable to the immobile crude oil reservoirs located in shallow deposits with minimal overburden (<45 m) present at the AOS (AOSTRA, 1990; Speight, 1999).

There are an increasing number of companies that are either already involved in commercial production, undergoing expansion, or in the process of acquiring land and operating licenses for surface mining, upgrading, and other *in situ* projects in the AOS region. Suncor and Syncrude are the two pioneering companies that began their surface mining extraction operations in 1967 and 1968, respectively (Speight, 2000). Over the last several years, the AOS has attracted other Canadian and multinational companies, including but not limited to Canadian Natural Resources Limited (CNRL), Total E&P Canada (Total), Petro-Canada, Imperial Oil, Shell Canada, Synenco, ConocoPhillips, EnCana, Japan Canada Oil Sands (JACOS), Devon Energy, Connacher Oil and Gas, and Nexen. In 2009, oil sands investment peaked at 20.7 billion dollars, and in 2010 there were approximately 91 active oil sands projects that were using various in situ recovery techniques, producing bitumen, under construction, or approved to produce bitumen in the future. Approximately 170 billion dollars in oil sands related projects are currently underway or proposed (Government of Alberta, 2011).

Syncrude Canada Ltd. and Suncor Energy Inc. are the two major oil sands developers in the AOS region that use the open-pit mining method. Both operators extract bitumen from the oil sands ore to produce synthetic crude oil using energy (mixers, heat), water, and process aids (e.g., NaOH, methyl isobutyl carbinol (MIBC)/kerosene surfactants) (MacKinnon et al., 2005). After mining, the extraction process involves conditioning, separation, and scavenging. The extraction process used by Syncrude and Suncor is the Clark hot water extraction process, and is based on gravity separation of bitumen from sand with the addition of air to promote bitumen flotation (Clark and Pasternack, 1932; Schramm et al., 2000). The extracted bitumen is then upgraded, converting it from viscous oil to lighter, more useable liquid end products. This synthetic crude oil can then be refined by typical refinery systems used for conventional petroleum (Singh, 1985). The synthetic crude oil production from these two companies was expected to be 14, 000, 000 m^3 in 2003 (Rogers, 2003). Oil sands production of bitumen from all active companies in 2005 was estimated at 1 million barrels per day and projected to increase to 3 million barrels per day by 2020 and 5 million barrels per day by 2030 (projections based on high oil prices and an

economically attractive environment) (Scott et al., 2008). In 2009, bitumen production averaged 1.49 million barrels per day (Government of Alberta, 2011).

1.2.1 Mining

The two main operators at the AOS, Syncrude and Suncor, are currently using conventional open-pit, surface mining techniques to extract the oil sands ore from the natural environment. After the overburden layer is cleared, the oil sands ore is mined using giant mechanical shovels, conveyor belts, and trucks. The oil sands ore is then transported to the extraction/processing plant via pipelines. It is here that the bitumen is separated from the solids. Technical considerations for the mining process include oil sands ore accessibility (overburden thickness), percent bitumen saturation, and regional low winter temperatures.

The amount of overburden covering the oil sands ore varies from 0 to 750 m at the AOS (AOSTRA, 1990). However, conventional mining techniques are only economically feasible on deposits that lie under 76 m or less of overburden. Deposits located at a depth between 76 and 198 m are considered too deep for surface mining, but not deep enough for *in situ* (non-mining) recovery techniques such as thermal and non-thermal strategies (Brient et al., 2000). Deposits below 198 m of overburden are recoverable using *in situ* techniques, but not without considerable constraints. As a result, only approximately 10% of the in-place oil sands deposits at the AOS can be surface mined (Speight, 1999). On average, the thickness of the underlying oil sands strata at the AOS is approximately 45 m, with a bitumen content of 10.5% by weight (AOSTRA, 1990; Rogers, 2003). For oil sands with a bitumen content of 10% by weight, it requires the processing of 12.5 metric tons of oil sand ore to recover 1 m^3 (6.3 barrels) of bitumen. Thus, below a certain bitumen concentration, the process is no longer considered to be economic. This value usually falls around 6% by weight (Speight, 2000).

The oil sands are a dense, solid material that are readily accessible during the summer. The majority of the AOS deposit is located between latitudes 55°N and 58°N (Adams et al., 2004) where temperatures can drop below -45°C in the winter. Temperature is a factor affecting the liberation and transport of oil sands ore to the extraction facility related to changes in viscosity and electrostatic repulsion between the sand and bitumen (Dai and Chung, 1995). Consequently,

considerations must be made to ensure that mining proceeds faster than the rate of frost penetration during winter. Supplemental measures such as blasting may also be required to dig through the frozen, concrete-like oil sands (Speight, 2000).

1.2.2 Extraction

Once the oil sand ore is mined, it is transported to an extraction plant where the bitumen is separated from the sand using a caustic hot water flotation method. The Clark hot water extraction process is named after Dr. Karl Clark who pioneered the procedure in the 1920s (Clark and Pasternack, 1932; Chilingarian and Yen, 1978). The high efficiency of this process (approximately 90%) can be attributed to the thin film of water surrounding the individual sand grains (AOSTRA, 1989), the density differences between bitumen and water, the addition of a caustic soda, and the presence of natural surfactants (Madill et al., 2001; Rogers, 2003). A thin film of water surrounds the individual sand grains around which the bitumen fills most of the remaining void space (Takamura, 1982). As a result, most of the bitumen is not in direct contact with the mineral phase providing the necessary environment for efficient hot water extraction. This water-wet sand is unique to the AOS which differentiates it from the oil-wet sands of the United States that are more difficult to separate (Schramm et al., 2000).

The three main steps of the hot water extraction process are conditioning, separation, and scavenging. The first step of extraction is conditioning. The mined oil sands are mixed with hot water (80 to 90 °C) and sodium hydroxide (NaOH) (50 to 200 g/t of oil sands) (Yen et al., 2004) to produce a slurry contained within a conditioning drum or tumbler. At the elevated temperature, a maximum density difference between the bitumen and water is established and bitumen that was heavier than water at ambient temperatures now becomes lighter and floats to the top (Speight, 1999). The caustic soda (NaOH) is added as an additional surfactant to promote the separation between sand and bitumen (Dai and Chung, 1995), and the tumbling action promotes aeration which is required for the formation of froth in the next step. The slurry from the tumbler is discharged onto vibrating screens where oversized materials such as rocks and lumps of clay are rejected before the slurry is pumped into the primary separation vessel (Speight, 1999).

The second step of extraction is separation. In the primary separation vessel, the sand settles to the bottom and the bitumen floats to the top as froth. The alkaline environment (pH approx 8.0 to 8.5) (Speight, 2000) created with the addition of NaOH in the previous step, solubilizes the natural surfactants such as NAs (Quagraine et al., 2005b). The surfactants act to increase the interfacial charges between the bitumen and solids, promoting bitumen detachment from the sand particles. The surfactants also lower the interfacial tension between the bitumen and air bubbles favouring bitumen attachment to the air and the subsequent formation of froth (Schramm and Smith, 1985). The froth containing the bitumen is swept off and collected, and the settled sand called tailings, is removed as waste.

The suspended fines, water, and residual bitumen referred to as middlings are purged from the primary separation vessel and scavenged for residual bitumen by the secondary separation vessel. This is the third step in the extraction process and is commonly referred to as scavenging. As before, the residual bitumen is extracted as froth. The bitumen froth from both the primary and secondary separation vessels is de-aerated and transported to the froth treatment plant which removes the water and remaining solids from the bitumen (Schramm et al., 2000; Rogers, 2003). The bitumen can now be upgraded to synthetic crude oil.

1.2.3 Upgrading

The process of upgrading the extracted bitumen into synthetic crude oil (Syncrude Sweet Blend (SSB) at Syncrude Canada Ltd.; and a similar sweet blend plus a sour blend containing higher amounts of sulfur at Suncor Energy Inc.) involves converting the viscous, heavy, tar-like oil to a lighter product that is more readily processed (Schramm et al., 2000; Squires, 2005). One of the methods used to accomplish this is called coking. As the carbon to hydrogen ratio of the oil is decreased, more energy is "packed" into the synthesized product which is lighter and has a lower boiling point (Singh, 1985). Syncrude uses a technique called fluid coking and hydrodesulfurization. Preheated bitumen is sprayed onto a hot bed of coke, which causes the heavy molecules to crack, producing light molecules in the form of vapour (Squires, 2005). The vapours are collected and separated by distillation into four main components: light process gas, naphtha (which is further upgraded into gasoline), kerosene, and gas-oil (Squires, 2005).

1.3 Liquid Tailings and Oil Sands Process-Affected Waters

During the production of synthetic crude oil large volumes of solid and liquid wastes are generated. Solid wastes include, but are not limited to, overburden, sand from the extraction process, and coke and sulfur from the coking process. Liquid wastes, created mainly during the extraction process, are called fluid tailings or liquid tailings and are the focus of this research.

One of the main problems resulting from the hot water extraction process is the disposal and control of enormous amounts of liquid tailings. A large quantity of water, referred to as process water, is required in the production process. Previous estimates suggest that the process requires about 15 m^3 of water to produce 1 m^3 of synthetic crude oil (MacKinnon, 1989). However as of 2010, it is estimated that only about 15 to 20% of this total water is drawn from local surface waters (up to 80% of water is recycled from the liquid tailings located in dedicated settling basins) and thus, the more integrated and efficient surface mining operations at the AOS uses approximately 2 to 2.5 m^3 of fresh water to produce 1 m^3 of synthetic crude oil (W. Zubot, Syncrude Canada Ltd., Edmonton, AB, Canada, personal communication). Each ton of oil sand in place has a volume of 0.45 m^3, which generates 0.65 m^3 of wastewater (Lai et al., 1996). This increase in volume is primarily due to the additional water used in the extraction process (0.6 to 0.7 m^3 water per ton of oil sand) (Hadwin et al., 2006). Only about 10% of the weight of mined oil sands ore is recovered (i.e., bitumen), the remaining material, along with added process water is called tailings. These tailings are further separated as liquid tailings and sand (Rogers, 2003). Liquid tailings have a pH between 8 and 9, and is a mixture of waste water, fine tails (water and solids), non-extractable bitumen (50:50:1), and other organic and inorganic components residual from the extraction process (MacKinnon, 1989; Herman et al., 1993; Young et al., 2007).

Due to environmental regulations and the industry's "zero discharge" policy (Quagraine et al., 2005b), discharge of any tailings into natural water bodies off-site is prevented. Tailings and tailings materials are currently stored on-site in designated retention ponds (MacKinnon, 1989) with the understanding that eventual reclamation of these materials must be undertaken. Daily tailings output from the AOS was estimated to be 300,000 m^3 by Rogers (2003).

1.3.1 Oil Sands Process-Affected Waters

The definition of process-affected waters includes: extraction waters in tailings; surface water in the tailings ponds; pore water in the sludge or tailings; water contained in the sand (above-grade tailings deposits) used to build dykes and beaches; and, the seepage water that is generated, collected and returned to the tailings ponds via engineering means (MacKinnon et al., 2005). This thesis collectively refers to this water as oil sands process-affected waters or OSPW. The more saline, softer process water displays a distinctive fingerprint of properties that are different from those of natural surface and ground waters of the area including elevated concentrations of sodium, chlorine, sulfate, carbonate and bicarbonate, boron, and NAs (MacKinnon et al., 2005). By 2003, Syncrude alone had produced greater than 500 million m^3 of process waters held within the various sinks on-site (MacKinnon et al., 2005). As of 2007, greater than one billion m^3 of OSPW, as water held within various tailings deposits, is stored within the AOS by Syncrude Canada Ltd. (Zubot, 2010). Plans to expand production and to open new mines in the region will inevitably increase the volume of OSPW generated and stored.

1.3.2 Contaminants in Oil Sands Process-Affected Waters

During the processing of oil sands, changes in water properties occur as dissolved constituents are leached from the ore and altered by extraction conditions and process aids (MacKinnon et al., 2005). The general classes of contaminants present in OSPW include suspended solids, dissolved solids, phenols, ammonia, oils, polycyclic aromatic hydrocarbons (PAHs), sulfides, trace metals, heavy metals and other inorganic and organic compounds (e.g., NAs) (Lai et al., 1996; Siwik et al., 2000; Speight, 2000; Squires, 2005; Gentes, 2006). Although OSPW contains a complex mixture of organic and inorganic constituents, existing evidence suggests that NAs comprise the most toxic portion to aquatic organisms (MacKinnon and Boerger, 1986; Verbeek, 1994; Lai et al., 1996; McMartin, 2003; Quagraine et al., 2005b). These dissolved organic acids are of particular concern, because of their persistence in the environment and their associated acute and chronic aquatic toxicity (Headley and McMartin, 2004; Clemente and Fedorak, 2005).

1.4 Naphthenic Acids

Heavy oil reserves, including bitumen, originate from fluid crude oils which have been degraded (decrease in the amounts of light and less complex components) in the reservoir by a

combination of biodegradation, thermal degradation, water washing, loss of volatiles, and inorganic oxidation (Tissot and Welte, 1984; Meredith et al., 2000). It is hypothesized that the origin of bitumen at the AOS is from the rich organic deposits of a previous marine environment, whose subsequent degradation of petroleum hydrocarbons, has lead to the formation of carboxylic acids including NAs (Chilingarian and Yen, 1978; Watson et al., 2002; Clemente and Fedorak, 2005). The end characteristics and concentrations of these acids present in bitumen are said to reflect both pre- and post-depositional environments. Naphthenic acids, a natural component of bitumen, are comprised of a large collection of saturated aliphatic and alicyclic carboxylic acids (Seifert et al., 1969; Fan, 1991; Headley and McMartin, 2004; Clemente and Fedorak, 2005). The NAs content in crude oil can vary from 0 to 4% (w/w) depending on the geographical location of the petroleum reserve (Dzidic et al., 1988). Their content ranges between 1 and 2% in the AOS (AOSTRA, 1989). In un-refined Athabasca bitumen, the carboxylic acid fraction is about 2% for which, according to earlier estimates, approximately 90% is comprised of the tricyclic acids that primarily make up the naphthenic acids fraction (AOSTRA, 1989). However, with recent advancements in NAs analysis, studies have shown that the classical and oxy-NAs only comprise <50% of the total abundance of compounds in extracted and isolated NAs fractions (Grewer et al., 2010).

Naphthenic acids are solubilized in OSPW during the alkaline hot water extraction process. The addition of caustic and the alkaline conditions (pH between 8 and 9) liberates the NAs, whose pK_a is about 5 (Rogers, 2003). Natural surfactants, including NAs, increase the interfacial charges between bitumen droplets and the solid particles (i.e., promote bitumen detachment), and decrease the interfacial tension of bitumen and the air bubbles (i.e., favour the attachment of bitumen to air) thus optimizing the primary separation process (FTFC, 1995a). Under these conditions, NAs are present as sodium naphthenates in dissolved form and remain in the aqueous compartments (e.g., water column and pore water) of process waters and fluid tailings (Leung et al., 2001). Clemente (2004) determined the average content of NAs in AOS ore samples from Syncrude to be 200 mg/kg of ore. In other words, the operations at Syncrude that process an estimated 500,000 tons of oil sands ore per day would result in a potential release of 100 tons of naphthenic acids each day (Clemente and Fedorak, 2005). The problem is further exacerbated when the tailings pond water is recycled for use as process water in the extraction process

(MacKinnon et al., 2005). The NAs are thus concentrated in the tailings pond over time, which can have final concentrations ranging from 20 to 120 mg/L (Clemente and Fedorak, 2005).

1.4.1 Chemical and Physical Properties

Naphthenic acids are a complex mixture of non-cyclic and mono- and poly-cyclic alkanes that contain carboxylated aliphatic side chains of various lengths. They are commonly characterized by the general chemical formula $C_nH_{2n+z}O_2$ where n indicates the carbon number, and Z represents the homologous group series number (i.e., the number of hydrogen atoms that are lost as the structures become more compact or as the fused-ring number increases). Moreover, cyclic NAs are also believed to be substituted with alkyl groups, usually a small methyl or ethyl group, denoted by the letter *R*. For example, the *Z* value is equal to 0 for saturated hydrocarbon chains, and becomes -2 in monocyclic NAs, -4 in bicyclic, and -6 in tricyclic, etc., (Lai et al., 1996; CEATAG, 1998; Schramm et al., 2000; Scott et al., 2005). Typical structures of NAs along with their *Z* series are shown in Figure 1.1.

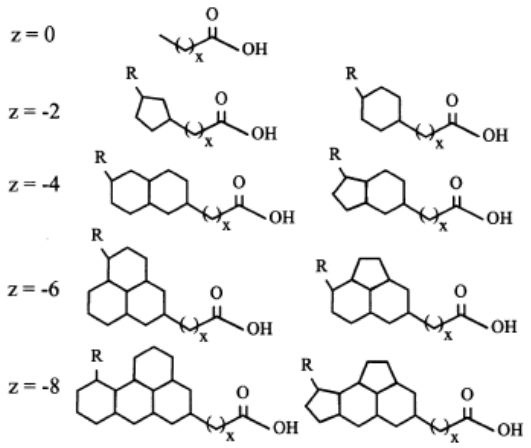


Figure 1.1. Typical structures of naphthenic acids, where *R* is an alkyl group and *n* is a variable describing the carboxyl side-chain length. Adapted from McMartin, 2003.

The complex mixture of NAs consists of hundreds of individual NA compounds with a wide range of molecular weights. Even within a specific group of carbon number and *Z* series, there can be numerous isomers each having a similar set of physical and chemical properties, making

them difficult to analyze and study individually with conventional analytical techniques (Holowenko et al., 2002). On a macro scale, characterization of groups of NAs mixtures is possible and the physico-chemical properties are specific to the geographical source (migration) and maturity (biodegradation) of NAs (Fan, 1991; Hsu et al., 2000). For example, Watson et al. (2002) highlighted some important studies that involved the application of carboxylic acid markers as indicators of biodegradation and migration of crude oils.

In general, NAs are chemically stable, non-volatile, highly viscous liquids with a boiling point ranging between 250 to 350 °C. They are completely soluble in organic solvents and oils, and are relatively water soluble (<50 mg/L at pH 7). Chemically, NAs behave like typical carboxylic acids (weak organic acids) with dissociation constants between 10^{-5} and 10^{-6} (pK_a between 5 and 6) and, as such, their solubility in water is greatly dependent on the ambient pH, being greater at higher pH values (Brient et al., 2000; Headley et al., 2002a; McMartin, 2003; Rogers, 2003; Clemente and Fedorak, 2005). Moreover, amphipathic NAs (i.e., having both a hydrophilic and a hydrophobic region) have properties consistent with natural surfactants, such as polarized aggregate arrangement, micelle formation, and the ability to reduce liquid surface tension, allowing them to persist at concentrations greater than 100 mg/L in process-affected waters (McMartin, 2003; Rogers, 2003). The surfactant nature of NAs may also be related to one of the proposed toxic mechanisms of action to aquatic organisms including membrane disruption and cytotoxicity (Quagraine et al., 2005b).

The majority of NAs from fresh tailings water from the Mildred Lake Settling Basin (MLSB) at Syncrude, were determined to consist of 2- and 3-ringed (Z = -4 and -6) compounds, with carbon numbers of 14 to 19 and molecular weights ranging from 220 to 298, as measured by gas chromatography-mass spectrometry (GC-MS) analysis (Holowenko et al., 2002; Rogers et al., 2002a; Clemente et al., 2003a; McMartin et al., 2004). However, the exact NAs composition of the mixture is source specific. A comparative study by Clemente et al., (2003a) revealed that the concentrations and complexity of NAs from tailings pond water, oil sands ore, and commercial preparations were all highly varied. Differences can be attributed to the source of the original bitumen, differences in the bitumen extraction process used, subsequent purifications, and natural degradation over space and time. For this reason, it is imperative that research always

include the source of NAs being investigated so that results can be compared across studies and interpreted correctly without confusion.

1.4.2 Environmental Fate, Distribution, and Bioaccumulation

Naphthenic acids are rarely present in aquatic environments naturally, although under certain conditions such as those present at the AOS, petroleum deposits may come in contact with surface water bodies and groundwater aquifers releasing NAs into natural systems. Over significant geological time, the Athabasca River has created valleys that cut through the shallow oil sands deposit (Schramm et al., 2000). As a result, runoff water from precipitation or riverbank erosion, that allows direct contact with the river, has washed NAs into the surface water at concentrations below 1 to 2 mg/L (FTFC, 1995b; CEATAG, 1998; Leung et al., 2003). Concentrations of NAs in near surface groundwater aquifers in contact with petroleum deposits have been measured in the range of <2 to 20 mg/L (CEATAG, 1998) and, similarly, the pore water of the Athabasca river may contain 6 to 8.5 mg/L of NAs (FTFC, 1995b). By far, the greatest potential for release of NAs into aquatic environments comes from anthropogenic sources, namely the development of oil sands such as those at the AOS. A number of physical, chemical and biological processes may affect the fate and bioavailability of NAs in aquatic environments impacted by oil sands operations.

As described previously, NAs are present in OSPW in carboxylate form as sodium naphthenates. The OSPW is stored on-site in tailings ponds and the NAs may be associated with the surface waters and fine tails for extended periods of time, especially if the tailings ponds are a part of future reclamation strategies. The ambient pH of OSPW is between 8 and 9. Under these alkaline conditions, NAs are non-volatile, have a relatively low fugacity, adsorption coefficient (K_d), and log octanol-water coefficient (log K_{ow}), and thus persist in the water column in their highly soluble, dissociated form (Schramm et al., 2000; McMartin, 2003; Rogers, 2003; Clemente and Fedorak, 2005). There is also the potential for leaks, spills, and seepage to occur from the storage ponds, which can result in the contamination of surrounding aquatic environments including groundwater (Bataineh et al., 2006). Clemente and Fedorak (2005) reported NAs concentrations ranging from 0.4 to 51 mg/L in groundwater samples taken near oil sands tailings ponds.

The surfactant nature of NAs would suggest that some of them would be distributed at the aqueous/non-aqueous interface between water and fines (i.e., clay and silt) (FTFC, 1995b) and, in the past, model NAs have been shown to adsorb to clays (Zou et al., 1997) via hydrogen bonding, electrostatic-dipole and van der Waal's interactions (Headley and McMartin, 2004). In general, the degree of adsorption of organic compounds is proportional to the fraction of organic carbon in the soil (i.e., silts and clays) and inversely proportional to the water solubility (Fetter, 2001).

A study conducted by Peng et al. (2002) showed relatively low adsorption coefficients for model single-ring NAs (K_d ranging from 0.22 mL/g to 0.10 mL/g) with the highest values associated with low pH, high dissolved ion content (decrease in solubility), and a more complex molecular structure of the sorbate (NAs). The generally low K_d values in this study imply that there would be little adsorption to soils resulting in high partitioning to the water column. A similar study conducted by Janfada et al. (2006) using NAs derived from oil sands tailings water revealed comparatively higher K_d values ranging from 1.3 mL/g to 17.8 mL/g. As in the previous study, the higher K_d values were associated with high dissolved ion content at an environmentally relevant pH of 8. Selective sorption of NAs based on chemical structure was also observed where NAs in a specific *Z* family with components in the carbon number range of approximately 13 to 17 tended to sorb more readily than the other congeners. Additionally, this study was able to show that soils with higher organic content resulted in greater levels of NAs sorption, especially in the presence of inorganic salts. In contrast to the previous study by Peng et al. (2002), Janfada et al. (2006) noted that oil sands NAs sorb strongly to soils, that their partitioning into the water column would be low, and that natural attenuation could be an important mechanism, especially in groundwater.

Exactly how much sorption takes place at the soil/water interface is debatable, but due to the ambient pH of the tailings pond water (between 8 and 9), and the high solubility (pK_a between 5 and 6) and carboxylate nature of the NAs in the aqueous phase, this tendency is expected to be quite low (Rogers, 2003). The majority of the NAs in the tailings pond remain "free" or distributed in the water column as opposed to being "bound" or adsorbed at the solid/solution interface (Schramm et al., 1984). This is consistent with what is observed in the field where

"free" NAs are the most bioavailable to cause toxicity in aquatic organisms and for the degradation and transformation processes involving microbes.

In view of the physical and chemical characteristics of NAs, most of the research has focused on biodegradation and the biotic processes that would act to reduce the concentrations of NAs in the water column as opposed to the abiotic processes (oxidation-reduction, sorption, hydrolysis, dissociation, photolytic, ozonation, etc.) (FTFC, 1995b; Rogers, 2003; Scott et al., 2008; Martin et al., 2010). Having a low Henry's constant (estimated) that governs the low volatility of the complex mixture (Rogers, 2003), combined with evidence of minimal photolytic degradation (McMartin et al., 2004), NAs would remain in the water column unless they were affected by microbial degradation, an important pathway for the removal of dissolved organic contaminants. In general, NAs are not expected to be very persistent in the water column due to the presence of indigenous microbial communities in tailings ponds capable of degrading NAs (Foght et al., 1985; Herman et al., 1994b; Del Rio et al., 2006). Under natural conditions NAs appear to be degrading over time, however, complete microbial mineralization has not been occurring and a portion of the NAs mixture, particularly the heavier molecular weight NAs, appear to be recalcitrant and resistant to further degradation (Quagraine et al., 2005b). Even after a period of 7 to 11 years, with no subsequent input of tailings water, and conditions specific to the substrate material and type of overlying water, the total NAs concentrations in some ponds had not decreased below 36 to 19 mg/L (Leung et al., 2001; Holowenko et al., 2002). This resistance to biological degradation may be related to the surfactant nature of the NAs, similar to what is proposed as a mechanism for aquatic toxicity (Headley and McMartin, 2004).

The potential for NAs bioaccumulation in aquatic organisms is expected to be relatively low with estimated log K_{ow}s in the range of 0 to 1 at ambient pH levels of 7.5 to 9 (Schramm et al., 2000). Research identifying the degree to which NAs bioaccumulate in aquatic organisms has been hampered by the lack of adequate analytical techniques required to examine NAs from plant and animal tissues (Gentes et al., 2006). A lack of specificity of the method is one noted problem (Young et al., 2007), largely due to interferences in quantitative and qualitative analyses by natural fatty acids (i.e., fats and oils) and co-extractives such as humic and fulvic acids that are chemically similar to NAs (Headley and McMartin, 2004). The evidence supporting NAs

bioaccumulation in animals has been mostly symptomatic where invertebrates, fish, rats, or birds exposed to naphthenic acids or OSPW have shown changes to various tissues, organs, hormones, and detoxification enzymes (Dokholyan and Magomedov, 1983; Nix and Martin, 1992; Siwik et al., 2000; Smits et al., 2000; Rogers et al., 2002b; Gentes et al., 2006; Gentes, 2006; Nero et al., 2006a; Nero et al., 2006b). Detailed investigations exploring the exact location and concentration of NAs within the animal have not been reported, but work with respect to tracking radio-labelled NAs and using stable isotopes to trace bacterial degradation has been promising (Videla et al., 2009).

Nevertheless, as referenced in a government report (FTFC, 1995b), evidence suggests that the bioaccumulation of total extractable hydrocarbons (TEH), which include NAs as a subcomponent, can occur within insect larvae. Further transfer into the food web is unknown, but studies using tree swallows that nest and feed (insects of aquatic origin accounted for 84% of their diet) near reclaimed oil sands impacted wetlands showed increased hepatic ethoxyresorufin-*o*-deethylase (EROD) activity, and increased plasma triiodothyronine (T3) concentrations and thyroxine (T4) content within thyroid glands proportional to the degree of wetland contamination (Smits et al., 2000; Gentes et al., 2006; Gentes et al., 2007a). However, nestling growth and survival, reproductive performance, hematocrit, blood chemistry, organ weights and specific aspects of immune function remained unaffected by exposure to NAs and OSPW in reclaimed wetlands (Smits et al., 2000; Gentes et al., 2007b).

The increase in hepatic EROD activity was presumed to be a result of the stimulation of hepatic detoxification enzymes responsible for the breakdown of toxic compounds linked to the presence of PAHs and NAs in the food items of these tree swallows (Smits et al., 2000). Similarly, the increase in thyroid hormones was presumed to be the result of the combination of OSPW contaminants present in the food and other environmental factors (Gentes et al., 2007b). It is important to mention that no direct evidence of NAs accumulation was identified nor measured in these studies, but rather inferred. Although it is possible that some of the observed effects may have been caused by the presence of PAHs, NAs or other constituents in OSPW (Gentes et al., 2006; Gentes et al., 2007a), it is also possible that these effects may be related to other

aspects such as weather, the availability of food resources, dietary composition, or the combination of all aforementioned factors (Gentes et al., 2007a; Harms et al., 2010).

It is possible that NAs could be accumulated by plants, but like the studies involving animals, direct measurement of NAs concentrations in plant tissues is rarely performed and reported due to the lack of a reliable analytical method and the presence of interfering compounds. Previously, Kamaluddin and Zwiazek (2002) attempted to measure the uptake of NAs in aspen seedlings. They examined NAs uptake in the xylem exudates of roots exposed to solutions containing NAs concentrations of 150 and 300 mg/L. The analysis of xylem sap revealed mean concentrations of 15 and 21 mg/L respectively, for the two exposure treatments. A more recent study by Armstrong et al. (2008) measured both OSPW and commercial NAs concentrations dissipation from hydroponic systems containing emergent aquatic macrophytes (cattails, common reed grass, and hard stem bulrush). They showed that commercial NAs dissipated from the hydroponic medium, whereas the OSPW NAs did not. The change in NAs concentration in the hydroponic medium can not definitively be associated with direct uptake of NAs in plants because the levels of NAs in plant tissue were not measured. The degradation of NAs could be due to one or more of the following processes: loss due to adsorption; absorption into plant roots; and, metabolism of NAs by the micro-organisms associated with the roots. Naphthenic acids degrading bacteria can be found in the sediment, at the sediment-water interface, and in biofilms attached to surfaces such as plant stalks and detritus. Therefore, it may be difficult to differentiate between NAs uptake and bacterial degradation in these types of studies where macrophytes such as cattails can provide increased surface area for biofilm development thus enhancing the NAs degradation rate mistakenly associated with uptake (Elshayeb, 2006; Biryukova et al., 2007).

Recent analytical advancements have allowed for the direct detection of NAs in both fish and plant tissue. Young et al. (2007) was the first to demonstrate that NAs could be detected in fish using the gas chromatography-mass spectrometry (GC-MS) method adapted from Merlin et al. (2007). Both commercial and oil sands NAs were qualitatively detected in homogenized fish fillets and viscera. This study only detected the presence of specific N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) derivitized NAs (e.g., NAs with 13 carbon

atoms and two rings; Z = -4 that yield m/z = 267 ions in reconstructed ion chromatograms) and thus is not considered to be quantitative. This method was further developed to quantitatively measure NAs concentrations in the flesh of both laboratory-exposed fish and wild fish collected from different surface water bodies in the vicinity of the AOS (Young et al., 2008), and then later to examine the distribution of NAs in various tissues such as gills, heart, liver, kidney, muscle, and eggs (Young et al., 2011). However, estimated NAs concentrations in fish tissues using this low resolution GC-MS method is subject to high variability and is specific to detecting free (unconjugated) NAs only. Nevertheless, it was shown that some fish tissues tend to accumulate more unconjugated NAs than others (Young et al., 2011). Currently, there is only one published method for the identification of oil sands NAs in plant tissue. Headley et al. (2011) successfully identified the presence of specific components (diagnostic ions at m/z 195, 247, and 277) in extracts of alder (*Alnus* spp.) saplings spiked with oil sands NAs using an ultra-high-resolution Orbitrap mass spectrometer. Although this technique is mostly diagnostic in nature, it is considered to be the first step towards the full quantification of NAs in plant tissue (Headley et al., 2011).

1.4.3 Microbial Transformation and Degradation

Although NAs can be degraded via aerobic and anaerobic pathways, the aerobic biodegradation of naphthenic acids in aquatic environments (field and laboratory) has been demonstrated as the more efficient metabolic pathway, and one that can be enhanced with an increase in dissolved oxygen (DO) (Quagraine et al., 2005a). Headley et al. (2002b) showed that of temperature, pH, and dissolved organic carbon, temperature had the most significant effect on biodegradation kinetics with increased rates between 10 and 30°C. A study by Lai et al. (1996) investigated the effects of temperature, DO, and phosphate concentrations on the degradation of radio-labelled NAs in oil sands wastewater microcosms. Phosphate addition was shown to increase the rate of NAs degradation in tailings pond water, while decreasing the DO concentration and temperature slowed the rate of degradation. It was concluded that DO and temperature were more important than phosphorus availability in controlling the biodegradation rates. Herman et al. (1993) noted that microbial activity in oil sands tailings was both nitrogen- and phosphorus-limited, which is consistent with the previous finding.

Naturally occurring microorganisms present in the sediments and tailings pond water column are capable of mineralizing commercial and oil sands NAs (Herman et al., 1994b; Lai et al., 1996; Clemente et al., 2004; Scott et al., 2005; Del Rio et al., 2006). Differences in the microbial community composition of NAs tolerant species can vary with the total NAs concentration and salinity of OSPW (Leung et al., 2001; Leung et al., 2003), but a mixed and diverse assemblage is most effective at degrading NAs (Quagraine et al., 2005b). A list of some of the taxa of microorganisms associated with process-affected waters that have the potential ability to metabolize NAs is presented in a review by Quagraine et al. (2005b). The biodegradation of NAs has also been investigated in the laboratory using: model NAs; surrogate NAs; commercial NAs such as Merichem, Kodak, and Fluka; and, NAs isolated from oil sands wastewaters (Clemente and Fedorak, 2005).

Surrogate NAs were used to describe the biodegradation pathways of specific carboxylic acids. For example, Quagraine et al. (2005a) suggested that the preferred route for the microbial degradation of aliphatic and alicyclic carboxylic acid was via β-oxidation. Differences in chain length, chain branching, positions where alkyl groups are substituted on the cyclic ring, number of cyclic rings, and other factors may affect and result in the differences observed in biodegradation rates. In addition, NAs mixtures associated with the oil sands are a complex group of compounds with varying stereochemistries (Headley et al., 2002b). Their degradation is dependent on the structure of the individual compounds, which may also explain the differences in their relatively slower biodegradation rates as opposed to the more rapid biodegradation kinetics of commercial mixtures (MacKinnon et al., 2005; Scott et al., 2005).

Data obtained from GC-MS analyses of commercial naphthenic acids (e.g., Merichem and Kodak) and NAs from oil sands tailings water revealed that the commercial NAs have carbon numbers between 7 to 17 (low molecular weights), whereas NAs present in tailings water have carbon numbers that range from 7 to 28 (higher molecular weights) (Scott et al., 2005). Results from aerobic biodegradation studies performed by Clemente (2004) and summarized in the review by Clemente and Fedorak (2005) indicate that NAs with lower molecular weights and fewer rings are most susceptible to biodegradation. This is consistent with other studies, including the results presented by Lai et al. (1996) that demonstrated that the rate of

biodegradation decreases with increasing number of carbon atoms (>17) and increasing degree of cyclization (from Z = -2 to Z = -6). The study also showed that microbes indigenous to oil sand tailings can degrade carboxylated cycloalkanes, but compounds with methyl substituents on the ring were more resistant to microbial activity.

It is important to note that biodegradation studies using commercial NAs alone do not accurately reflect the biodegradability of NAs in OSPW. Organic acids found in oil sands tailings waters are less susceptible to biodegradation and detoxification than commercial mixtures of NAs, because commercial NAs generally have lower molecular masses and are less complex than NAs from process waters (Herman et al., 1994b; Scott et al., 2005). Scott et al. (2005) followed changes in NAs concentrations over time for mixtures of NAs from tailings pond waters and commercial NAs (Kodak) together. For the mixture, the results illustrated that the NAs with lower molecular masses (≤17) were preferentially biodegraded over the NAs from tailings water with higher molecular weights (≥18). After the initial degradation of the Kodak NAs, the higher molecular weight NAs of tailings water persisted at essentially constant concentrations over the remainder of the incubation period.

Previous studies have shown that on-site, aerobic, aquatic reclamation environments are capable of reducing the concentration of lower molecular weight NAs with ageing (10 to 15 years), but processes are slower and less complete with the heavier molecular weight NAs (Lai et al., 1996; Leung et al., 2001; Holowenko et al., 2002; Quagraine et al., 2005a; Quagraine et al., 2005b; Biryukova et al., 2007; Whitby, 2010). The biodegradation of OSPW NAs using laboratory incubations also showed selective degradation. High molecular weight NAs were found to be more resistant to microbial degradation than the low molecular weight NAs (Scott et al., 2005). Specifically, NAs with lower carbon numbers, were readily biodegradable compared to NAs with higher carbon numbers (Clemente et al., 2004; Del Rio et al., 2006) and NAs associated with the lower Z families or lower degrees of cyclization were the least persistent (Biryukova et al., 2007; Han et al., 2008). Other studies have either suggested or demonstrated NAs resistance to biodegradation due to: differences in structural conformation (Headley et al., 2002b); alkyl substitution (Bataineh et al., 2006); branching and location of branching on the cycloalkane ring (Han et al., 2008); side chain length and branching (Smith et al., 2008); and, oddness or evenness

of the carbon chain length (CEATAG, 1998). In general, Han et al. (2008) concluded that the resistance of NAs to biodegradation is related to (in decreasing order of importance) higher degrees of alkyl branching, higher *Z* series, and higher carbon number. The resistance to complete microbial degradation of OSPW NAs has also been attributed to the surfactant nature of the molecules, which is also related to the associated aquatic toxicity to microorganisms (Headley and McMartin, 2004).

There are a number of abiotic and emerging treatment processes that have been investigated for the degradation of NAs in OSPW. These include photolysis (McMartin et al., 2004), photocatylsis and microwave-assisted photocatalysis (Headley et al., 2010; Mishra et al., 2011), advanced oxidation processes using UV light (Liang et al., 2011), and ozonation (Scott et al., 2008; Martin et al., 2010). Ozonation appears to be the most promising treatment process as it targets the larger, more complex, persistent congeners in a given NAs mixture. Using a GC-MS method, Scott et al. (2008) demonstrated that ozonation decreases the average molecular size range of the components detected, and the total NAs concentration by approximately 70%. Ozonation also appears to accelerate natural NAs bioremediation processes even after mild or light ozonation of OSPW, suggesting that this treatment process could be complimentary to the biodegradation capacity of microorganisms already present in OSPW (Martin et al., 2010).

1.4.4 Aquatic Toxicity

Naphthenic acids composition and toxicity are source-specific and variability in test conditions can affect the bioavailability and toxicity of NAs to aquatic organisms (Rogers, 2003). It is important to note that in whole effluent studies where no NAs quantification was conducted, the biological responses observed can potentially be a result of the exposure to other contaminants present in the test waters. Factors such as DO, temperature, salinity, hardness, and the presence of additional contaminants may increase or decrease the sensitivity of biota to NAs, or cause toxicity themselves (CEATAG, 1998; Siwik et al., 2000). As a result, aquatic toxicity data and results should be interpreted with caution.

Although OSPW contains a complex mixture of organic and inorganic constituents, evidence suggests that NAs comprise the most toxic portion to many organisms (MacKinnon and Boerger,

1986; Verbeek, 1994; Holowenko et al., 2002; Headley and McMartin, 2004). Naphthenic acids have been shown to have inhibitory or toxic effects on a variety of organisms including plants, rats, fish, amphibians, snails, zooplankton, and bioluminescent bacteria (Patrick et al., 1968; Dokholyan and Magomedov, 1983; Uzhdavini and Glukharev Iu, 1984; Pollet and Bendell-Young, 2000; Leung et al., 2001; Holowenko et al., 2002; Kamaluddin and Zwiazek, 2002; Leung et al., 2003; Rogers, 2003; Clemente et al., 2004; Clemente and Fedorak, 2005). Due to the surfactant characteristics of NAs, the primary proposed toxic mechanism of action to aquatic organisms for NAs is believed to be general narcosis leading to membrane disruption and the disruption of osmotic homeostasis (Quagraine et al., 2005b; Frank et al., 2008).

In other studies, NAs have been suspected as endocrine-disrupting substances (Headley et al., 2002a; McMartin, 2003; Rogers, 2003; Hao et al., 2005; Quagraine et al., 2005b). Lister et al. (2008) found that goldfish, *Carassius auratas*, exposed to OSPW (NAs concentrations up to 24.1 mg/L) resulted in the disruption of normal endocrine functioning through alterations to both reproductive and glucocorticoid hormone biosynthesis. These effects included reduced plasma testosterone and 17β-estradiol concentrations, and increased plasma cortisol levels. However, when experiments were repeated using naphthenic acids extracts re-dissolved in control lake water (NAs concentrations up to 6.1 mg/L), similar effects on plasma steroid levels of *C. auratas* were not observed, suggesting that other constituents in OSPW may be responsible for effects on steroid biosynthesis. Exposure of the H295R cell line to OSPW also resulted in decreased testosterone production, however, an increase in 17β-estradiol concentrations (He et al., 2010) was in stark contrast to what has been observed *in vivo* for fish (van den Heuvel et al., 1999; Lister et al., 2008). He et al. (2010) suggest that this difference may be due to several factors including, but not limited to, the differences in the complexity of the systems (e.g., cells versus tissue) and inter-species differences (e.g., mammalian versus fish). Although the preceding studies implicate NAs as potential compounds involved in endocrine disruption, the exact constituents in OSPW responsible for these endocrine effects remain unidentified.

MacKinnon and Boerger (1986) measured the toxicity of oil sands tailings pond water containing NAs and found it was acutely toxic to rainbow trout (LD_{50} = 7% v/v). In yellow perch (*Perca flavescens*), sublethal effects due to NAs exposure resulted in the proliferation of epithelial,

chloride, and mucous cells of the gills (Nero et al., 2006a). The proliferation of these cells is possibly the result of irritation as a protective response of the gill to reduce toxicant entry. Comparable protective effects have been observed in rainbow trout (Partearroyo et al., 1992) and catfish (Roy, 1988) gills exposed to surfactants that have similar properties to NAs. The changes in gill structure following exposure to NAs may have consequences for other important physiological functions of the gills, namely a decrease in gas exchange efficiency, and may lead to long-term fish health issues (Nero et al., 2006a). Dokholyan and Magomedov (1983) studied acute toxicity in a number of fish species at different ages and determined LC_{50}s for NAs ranging from 25 to 75 mg/L. Sixty-day repeat exposure studies were also performed on several species of fish. Median lethal NAs concentrations (LC_{50}s) were 11, 2.0, 14, and 1.4 mg/L for sturgeon (*Acipenser gueldenstaedi*), Kutum (*Rutulis frisii kutum*), Roach (*Rutulis rutulis caspicus*), and Chum salmon (*Oncorhynchus keta*), respectively. However, the source of these NAs was not specified. Further evidence of acute toxicity to fish is described by Patrick et al. (1968) who observed a 96-hour LC_{50} of 5.6 mg/L commercial NAs for bluegill sunfish (*Lepomis macrochirus*), and Davis (1992) who observed a 96-hour LC_{50} of 5 mg/L commercial NAs in stickleback.

Due to its relatively low cost, speed, and reproducibility, the Microtox® bioassay using the bioluminescent bacteria *Vibrio fischeri* (formerly *Photobacterium phosphoreum*), is frequently used to monitor the toxicity of the oil sands tailings water, and surrogate and commercial NAs solutions (Holowenko et al., 2002; Clemente et al., 2004; Frank et al., 2008). MacKinnon and Boerger (1986) determined the toxicity of oil sands tailings water using *V. fischeri* and reported an IC_{50} between 20 and 30% v/v, and an IC_{20} of 10% v/v after 15 minutes of incubation. Using the Microtox® assay, Herman et al. (1994b) concluded that naphthenic acid sodium salts had an EC_{50} value of 30% v/v.

Some studies have shown that a decrease in total NAs concentration corresponds to a decrease in toxicity as measured using the Microtox® assay (MacKinnon and Boerger, 1986; Clemente et al., 2004; Del Rio et al., 2006). Herman et al. (1994b) also demonstrated that mineralization of oil sands NAs, via microbial activity, was possible and that this corresponded to a reduction in Microtox® toxicity. However, they found that some residual (i.e., chronic) toxicity persisted and

that this could potentially be related to recalcitrant NAs. Using GC-MS, Holowenko et al. (2002) were the first to describe the relationship between the increasing content of heavier molecular weight NAs (identified as the "C22+ cluster") within a given NAs mixture due to natural degradation, which coincided with a reduction in total NAs concentration and aquatic toxicity. In the field, the acute toxicity of aged OSPW held in various experimental test ponds on-site is generally removed within 1 to 2 years, whereas the disappearance of chronic toxicity takes approximately 2 to 3 years (Herman et al., 1994b). The biodegradative and toxicological properties of NAs mixtures are partly dependent on their differences in molecular composition, structure, and polarity. The relationship between the persistence, selective biodegradation, and associated toxicity of complex NAs mixtures remains unclear due to the lack of available analytical techniques required for the complete separation and identification of the numerous, closely related individual compounds.

1.4.5 Regulations and Criteria

Significant environmental and regulatory attention has been focused on the NAs fraction of oil sands material due to their persistence in the environment and their identified aquatic toxicity at the concentrations found in tailings pond waters (MacKinnon and Boerger, 1986). However, there are no ambient fresh water or marine water quality guidelines for NAs in Canada or the United States. This is primarily due to a lack of sufficient chronic toxicity data regarding the long-term exposure of freshwater aquatic biota (CEATAG, 1998). Also, NAs are a complex mixture of carboxylic acids, and the identification of what components of the naphthenic acids mixture are actually causing chronic toxicity remains unclear. That said, based on studies conducted by Dokholyan and Magomedov (1983), a maximum permissible limit of 0.15 mg/L of sodium naphthenate in sea water was established in the former USSR. The applicability of strict regulatory numerical guidelines with respect to NAs in the oil sands area, where significant natural background levels of NAs exist, is questionable pending accurate and consistent analytical measurement.

1.5 Naphthenic Acids Analytical Quantification and Characterization

Interest in the detailed analysis of the carboxylic acid fraction in crude oils and petroleum hydrocarbons, along with the first attempts to identify "naphthenic acids" as oil sands

components, has been around since the 1950s and 1960s as summarized by Seifert et al. (1969). The sophisticated analyses of these acidic components involved exhaustive extraction, separation, and chemical reaction steps prior to characterization by various spectroscopic techniques (Dzidic et al., 1988). Initially, the importance of NAs analysis was related to their corrosive nature in refinery systems (Derungs, 1956; Gutzeit, 1977; Dzidic et al., 1988; Turnbull et al., 1998; Hsu et al., 2000), and geochemical considerations for their use as markers with respect to petroleum origin, migration and biodegradation (Jaffe and Gardinali, 1990; Jaffe and Gallardo, 1993; Meredith et al., 2000; Watson et al., 2002). More recently, the presence of NAs in waste streams associated with the extraction and refinery operations, specifically the tailings of the oil sands industry, has resulted in an increased focus on the development of analytical methods to quantify and characterize NAs mixtures in aqueous solutions. Establishing more sensitive, robust analytical methods for the detection and characterization of NAs in abiotic and biotic environments has coincided with the increase in research regarding their environmental fate, persistence, biodegradation, and toxicity to aid in the development of successful reclamation strategies at the AOS.

Due to the complexity of NAs mixtures, the complete separation, quantification and identification of individual compounds is not possible with currently available analytical techniques. However, total concentrations can be measured in oil and water matrices by treating the NAs as a single group. To partly describe the composition of the complex NAs mixture, NAs can be divided into sub-groups, based on carbon number and molecular structure (i.e. ring structure) (Clemente and Fedorak, 2005). Analytical methods are based on the common principles of extraction, separation, and subsequent analysis by mass spectrometry. Although there have been various studies that have either improved on or provided an alternative to the basic method, this section highlights the most common and effective approaches to date used for both NAs quantification and characterization.

1.5.1 Quantification

Naphthenic acids have been measured in both petroleum and aqueous solutions. For example, the oil acidity or NAs concentrations in crude oils is measured by its total acid number (TAN), which is the number of milligrams of KOH required to neutralize the acidity of one gram of oil

(Meredith et al., 2000). Details of the methodology and, accuracy and precision data are provided in ASTM D664 (potentiometric determination) (ASTM Standard D664-09a, 2009) and ASTM D974 (calorimetric determination) (ASTM Standard D974-11, 2011). Other methodologies used to estimate acids in crude oil include gas chromatography (GC) with flame ionization detection (FID) (Mackenzie et al., 1983; Jaffe et al., 1988) and gas chromatography coupled with mass spectrometry (GC/MS) (Jones et al., 2001).

In aqueous solutions, the quantification of NAs has been conducted using GC with flame ionization detection (FID) (Herman et al., 1994b), gas chromatography in conjunction with low- and high-resolution mass spectrometry (GC-LRMS and GC-HRMS) (Merlin et al., 2007), electrospray ionization-mass spectrometry (ESI-MS) (Headley et al., 2002a; Lo et al., 2003) and high performance liquid chromatography (HPLC) mass spectrometry (Clemente et al., 2003b; Clemente et al., 2004; Yen et al., 2004; Scott et al., 2005). However, the Fourier-transform infrared (FTIR) spectroscopy method, developed by Jivraj et al. (1995) for Syncrude Canada Ltd., is the most commonly used method to determine total NAs concentrations in OSPW and natural waters. This method is considered the industry standard for NAs quantification and has been used in multiple studies since its inception (Holowenko et al., 2000; Holowenko et al., 2001, 2002; Kamaluddin and Zwiazek, 2002; Rogers et al., 2002a; Leung et al., 2003; Yen et al., 2004).

In short, naphthenic acids are extracted into an organic solvent such as dichloromethane (DCM) from an acidified aqueous sample and then concentrated and analyzed by FTIR spectroscopy. As described in detail by Rogers (2003), the FTIR uses infrared frequency radiation to induce vibrations of chemical bonds in molecules. Different bonds or functional groups have different resonant frequencies and the magnitude of the produced signal is proportional to the amount of radiation absorbed. The monomeric and dimeric forms of the carboxylic groups of NAs have absorbance peaks in the wavenumber region of 1740 to 1750 cm^{-1} and 1700 to 1715 cm^{-1}, respectively. Based on this fact, Jivraj et al. (1995) generated a standard calibration curve using commercially available NAs. Maximum resonant signals were observed at surprisingly similar wavenumbers of 1743 cm^{-1} (monomer) and 1706 cm^{-1} (dimer) for the commercially available Kodak^{TM} naphthenic acids. Thus, the sum of the absorbances at these two wavenumbers for the

sample being analysed is compared to those of the calibration curve providing a fairly accurate measure of total NAs concentration ranging between 0 to 500 mg/L (Rogers, 2003). Clemente and Fedorak (2005) stated that the minimum detection limit for this method is typically in the order of a few tenths of a milligram per litre, but can depend on the nature of the solvent extraction and concentration steps. Limitations of this method include the susceptibility to interferences from the presence of non-naphthenic acid carboxylic acids such as fatty acids (Brient et al., 2000), longer personnel time required for analysis compared to other available methods such as HPLC (Yen et al., 2004), and the lack of an ability to resolve carbon numbers or *Z* families to provide more structural information of specific homologues of NAs (Holowenko et al., 2002).

Based on the work of Miwa and colleagues as reviewed in Miwa (2000), who used HPLC to measure carboxylic acids in food and beverages, Clemente et al. (2003b) developed a simpler and faster method to measure NAs concentrations in aqueous mediums than the previous FTIR method. The authors derivatized the NAs from an aqueous sample with 2-nitrophenylhydrazine (NPH) in the presence of a coupling agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Elution of the derivatized compounds via HPLC resulted in an unresolved hump. The area of the unresolved hump for the sample was compared to the humps created using standard commercial NAs preparations, in order to monitor the biodegradation of NAs in the laboratory. The benefit of this method over the FTIR method is that it does not require the time consuming extraction step used to isolate the acids from the aqueous medium prior to analysis. Depending on the overall concentration of NAs present in the sample, large sample volumes may be required for this extraction step. Conversely, the HPLC method only requires the removal of about 3 mL of sample of which only 100 uL of centrifuged supernatant is used in the derivatization process whereby avoiding the use of a chlorinated solvent and saving preparation and analysis time (Clemente et al., 2003b).

Unfortunately this method has relatively high day-to-day variability and has a detection limit of about 15 mg/L (Clemente and Fedorak, 2005). Improvements to this method were made by modifying the concentrations and proportions of the aqueous sample, and the NPH and EDC reagents as outlined in Table 1 of Yen et al. (2004). With these changes the detection limit was

reduced to about 5 mg/L. This study also found that in OSPW samples, NAs concentrations reported using the FTIR method were on average 11% higher than the concentrations determined using the HPLC method, however, it could not be determined which method gave the more accurate number (Clemente and Fedorak, 2005).

1.5.2 Characterization

Much analytical effort has been devoted to characterizing the molecular composition of NAs because their corrosive (Turnbull et al., 1998), sorptive (Peng et al., 2002; Janfada et al., 2006), biodegradative (Herman et al., 1993; Lai et al., 1996; Headley et al., 2002b; Scott et al., 2005; Han et al., 2008), and toxicological (Herman et al., 1994b; Holowenko et al., 2002; Clemente et al., 2004; Lo et al., 2006) properties have all been shown to be partly dependent on differences in molecular composition, structure, and polarity. The acid character, and the numerous, closely related individual compounds (i.e., congeners) with small differences in polarity and volatility, have hampered the development of analytical techniques required for the complete separation and characterization of a given NAs mixture.

In spite of these challenges, mass spectrometry (MS) and/or gas chromatography with mass spectrometry (GC/MS) methods have provided the best information to date. In mass spectrometry, compounds are ionized into charged fragments which are then separated with a mass analyzer based on their nominal mass/charge (m/z) ratio. The relative abundance of all the charged fragments versus the nominal mass are plotted in a mass spectrum.

To determine the molecular composition of NAs, the general approach has been to assign carbon and Z numbers from mass spectra (Dzidic et al., 1988; Fan, 1991; St John et al., 1998). Naphthenic acid homologues are traditionally represented by the general formula $C_nH_{2n+z}O_2$ where, n indicates the carbon number, and Z specifies a homologous series. Based on this empirical formula, a table is generated containing the expected molecular masses for different groups of NAs isomers according to carbon number and Z series not unlike Table 1 presented in Clemente and Fedorak (2005). The m/z value of the charged fragments from a mass spectrum is used to assign each observed ion to the appropriate acid group in the table. For example, an ion with a m/z = 295 would be assigned to the naphthenic acid group with an expected molecular

mass = 296 which from Table 1 corresponds to the isomer having *n* = 19 (carbon number) and *Z* = -2 (structure with 1 ring). The intensity of the ion is entered into the corresponding acid group of this matrix. This is repeated for each ion observed in the mass spectrum and the overall matrix provides a summary of the relative distribution of the components in a given naphthenic acid sample.

One drawback of this method is the incomplete resolution of acid homologues. As expected, a number of isomers may be present in the same homologous *Z* series, and homologues in different *Z* series can have the same molecular weights but different formulas. For instance, homologues with the formula $C_{14}H_{26}O_2$ (*Z* = -2) have the same molecular mass as homologues with the formula $C_{15}H_{14}O_2$ (*Z* = -16), which may represent the presence of highly aromatic acids (Fan, 1991). Another drawback is that the absolute concentrations of groups of NAs can not be determined because the absolute response factor for each NA type is not available and the ion intensities for each acid group is relative to what is in the total sample (Fan, 1991).

Ionization or the generation of charged fragments is accomplished by either hard or soft ionization methods and several of these have been used with varying degrees of success to qualitatively determine the composition of NAs. Hard ionization techniques such as electron impact (EI) ionization usually results in extensive fragmentation of molecules and limits the amount of molecular mass and structural information that can be obtained (Skoog and Leary, 1992; St John et al., 1998). When dealing with complex mixtures such as NAs, the fragmentation patterns are obscured by ions from many different co-eluting acids and the identification of different acid types becomes difficult even by high-resolution gas chromatography (Dzidic et al., 1988).

Soft ionization techniques result in less fragmentation and have been used to determine the molecular composition of NAs in crude oils, OSPW, and commercial preparations. The spectra from these techniques are usually simplified and information on molecular structure and concentration can be obtained more readily (Skoog and Leary, 1992). Two important soft ionization techniques with respect to acid analysis are chemical ionization and fast atom bombardment.

Dzidic et al. (1988) used fluoride ion (FI) chemical ionization (FI-MS) to characterize NAs in several California crude oils and refinery wastewater. The NF_3 reagent gas used in this method yields fluoride ions (F^-) when impacted by the electron beam. The NAs (i.e. RCOOH) are selectively ionized by the F^- ions in an acid-base reaction to form the base $RCOO^-$ and the acid HF. Two important characteristics of this method that simplify the interpretation of the resulting mass spectra are that the non-acidic compounds such as hydrocarbons are not ionized by F^- ions and therefore do not interfere in the analysis, and only the single $RCOO^-$ carboxylate ions, without any fragmentation, appear on the mass spectra.

Fast atom bombardment mass spectrometry (FABMS) is well known for its ability to analyze polar, non-volatile and/or high molecular weight compounds (Skoog and Leary, 1992; Rogers, 2003). Fan (1991) used this technique in negative ion mode to analyze NAs in commercial preparations (Kodak^{TM} NAs) and in crude oils from the states of Louisiana, California and Montana. The samples, generally held in a condensed state using a neutral glycerol solution matrix, are ionized by bombardment with xenon or argon atoms (Skoog and Leary, 1992). In this case, NAs samples were contained in a more basic triethanolamine (TEA) matrix as opposed to glycerol, and bombarded by a beam of accelerated xenon atoms. Having a higher affinity for protons, the basic matrix absorbs the protons removed from the NAs via atom bombardment, resulting in the formation of non-fragmented $RCOO^-$ ions appearing on the mass spectra similar to the previous fluoride ion chemical ionization method. In this study, it was noted that the spectra obtained using the FABMS method showed a wider range of molecular weight distributions or carbon numbers (range up to C50) for a sample of crude oil than that of the spectra obtained using the CI method of Dzidic et al. (1988) (carbon number range up to C32). However, the overall relative mole percent distribution of *Z* groups of the entire sample was very similar between the CI and FABMS methods and both are equally capable of providing a valuable fingerprint (Fan, 1991).

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been used in a number of studies to determine the molecular distribution of NAs (Hsu et al., 2000; Rogers et al., 2002a; Rudzinski et al., 2002; Lo et al., 2003, 2006). In ESI, the sample is dissolved in a more volatile solvent in which the analyte, or NAs in this case, are present in the

dissociated form. As described in McMartin (2003) and Rogers et al. (2002a), an electrospray is generated by applying a voltage between the elecrospray probe and the sampling cone, that results in the formation of a mist of small droplets containing the analyte. The surface of the droplets containing the ionized compound will become either positively or negatively charged and the subsequent evaporation of the solvent from the droplets brings the charged analyte molecules closer together. The density of the charges at the droplet surface increases to a point where the droplet explodes. The released ion free of solvent is then filtered, focused and detected by the mass analyzer. Atmospheric pressure chemical ionization is essentially, a gas phase ionization as opposed to the liquid phase ionization as in ESI. In this method the sample, dissolved in a solvent and transferred to the tip of a heated nebulizer in a flowing carrier gas, is ionized by corona discharge to produce the primary ions. The ions in the gas are transferred into the mass analyzer by vacuum (Barker, 1999; Gross, 2004). For NAs, Lo et al. (2006) compared these two methods and found that APCI-MS was less sensitive to instrument settings (i.e. cone and extractor voltages) and gave a wider range of detection and quantitation than ESI-MS, but its detection limit was poorer, had higher operating costs, and required more frequent instrument maintenance. In general, the negative ion APCI, using acetonitrile as the solvent, results in less fragmentation and yields the cleanest spectra from the other alternatives including CI, ESI, and liquid secondary-ion mass spectrometry (LSIMS) (Hsu et al., 2000; Rudzinski et al., 2002).

Fourier transform ion cyclotron resonance mass spectrometry (FTICR) allows for ultra-high resolution and mass accuracy of the analyses with minimum sample volumes (Gross, 2004; Clemente and Fedorak, 2005). High resolution is important when resolving closely spaced signals, and mass accuracy is a measurement of how well the observed *m*/*z* correlates to the "true value", such as in the case of complex mixtures like NAs (Barrow et al., 2004). A description of the main features pertaining to the characterization of NAs is given in Barrow et al. (2004). In summary, the circular motion of ions in the presence of a magnetic field have an angular frequency, called the cyclotron frequency, that is related to the mass-to-charge ratio of the ion. Barrow et al. (2003) and (2004) used FTICR to demonstrate its unequivocal assignment of species to signals when characterizing NAs.

High-field asymmetric wave-form ion mobility spectrometry (FAIMS) is a recently developed technique used to separate ions at atmospheric pressure and room temperature. This technique involves the application of a high-voltage, high-frequency asymmetric waveform across parallel plates or concentric cylinders (Purves et al., 1998) in order to separate ions carried by a gas, based on the motion of ions induced by electric fields (Purves and Guevremont, 1999). The FAIMS device can be considered as an ion filter with the capability of selectively transmitting one type of ion out of a mixture (Guevremont and Purves, 1999) such as in NAs. Details of this method are summarized in Purves et al. (1998), but the basis of the separation of ions is in the difference of the mobility of an ion at high field relative to its mobility at low field. A compensation voltage (CV) is required to "balance" the ion within the electric field. To analyze a mixture of ions, the CV can be scanned to yield a CV spectrum.

High-field asymmetric wave-form ion mobility spectrometry can also be coupled to ESI and interfaced with a mass spectrometer (ESI-FAIMS-MS), which allows for separation and peak identification based more on ion structure rigidity than on ion size for the characterization of NAs mixtures (Gabryelski and Froese, 2003). It was shown in this study that sample dilution with methanol was sufficient to analyze the NAs directly thus eliminating the need for extensive sample preparation as opposed to the salt-to-acid conversion and derivatization steps required for GC/MS. Therefore, the effort required in collecting the same information with respect to carbon number and unsaturation factor for characterizing NAs is considerably smaller for ESI-FAIMS-MS than GC/MS. Moreover, Gabryelski and Froese (2003) reported strong evidence for the ability of FAIMS to separate structural isomers for an ion with a given *m*/*z*, the ability to determine the ring structure and character of non-polar substitution groups, and the identification of ion peaks in mass spectra corresponding to fragmentation products found in the other methods (i.e., ions easily dissociated in the mass spectrometer interface causing interference or false peaks during detection).

1.5.3 Challenges and Advancements

In the past, GC/MS has been used to elucidate structural features of carboxylic acids in crude oils to investigate their origins, maturation, migration and biodegradation (Mackenzie et al., 1983; Behar and Albrecht, 1984; Jaffe and Gardinali, 1990; Jaffe and Gallardo, 1993). Seifert and

Teeter (1969) used hard ionization techniques, like electron impact (EI) ionization to identify the presence of multiple compound classes of carboxylic acids present in crude oil. It was noted that derivatization of acids to esters resulted in a cleaner mass spectra with fewer overlapping peak regions providing a increased resolution for the identification of 2, 3, 4, and 5-ring carboxylic acids. The derivatization step converts the acids into an ester form (i.e., methylation) improving the volatility of the generally non-volatile, heavier molecular weight NAs which in turn, results in a cleaner fragmentation spectra (Rogers, 2003).

Perhaps the most accessible and most often used method for the characterization of NAs from the AOS is the GC/MS method based on the work of St. John et al. (1998) and Holowenko et al. (2002). This simpler method, initially developed as an alternative to FI-MS, analyzes the *tert.*-butyldimethylsilyl (t-BDMS) derivatives of NAs, unlike other methods that make esters of the acids. Derivatization is obtained by the reaction of N-methyl-N-(*tert.*butyldimethylsilyl)trifluoroacetamide (MTBSTFA) with the acidic proton on carboxylic acids. All previously described soft ionization MS techniques have allowed NAs composition analysis directly, without any derivatization and pre-separation steps, and resulted in minimal molecular ion fragmentation. Conversely, analysis by GC/MS in combination with electron impact (EI) requires chromatographic separation along with the t-BDMS derivatization, but similarly, allows for improved volatility along with a relatively stable derivative that displays minimal fragmentation (Holowenko et al., 2002; Rogers, 2003). Subsequent spectral interpretation produces information pertaining to the molecular mass and structure of NAs.

St. John et al. (1998) was also able to determine relative percent composition of the individual components within NAs mixtures by carbon and *Z* numbers, and presented this information in a table. Holowenko et al. (2002) used a similar table except that it excluded 47 entries which did not conform to the empirical formula of NAs. The data table was used to plot the relative abundances of component NAs, normalized to a summed total intensity value, as three dimensional bar graphs. This allowed for the comparison of compositional differences among several NAs mixture samples. In this plot, the bars (y-axis) represent the percentage (by number of ions) of NAs in the mixture that accounts for a given carbon number, *n*, (x-axis) of a given *Z* family (z-axis) corresponding to a specific *m*/*z* value from the GC/MS analysis. The sum of all

bars is equal to 100% and therefore, individual bars do not represent absolute concentrations, but are relative to what is in the total sample. Illustrating ion distributions in this fashion, commonly referred to as a NAs "fingerprint", provides a convenient approach to monitor the degradation of NAs and to identify differences in NAs composition of samples from different sources.

Other methods that have been used to present this same information include two dimensional graphs where the relative intensity of each ion is plotted versus the carbon number or molecular weight with a separate curve for each *Z* number (Fan, 1991; Hsu et al., 2000), and a pie chart to present the distribution of acids based on *Z* number (Rogers et al., 2002a). However, the three dimensional bar graphs appear to be the most widely used method; especially in studies monitoring the degradation and ageing of NAs; those highlighting the compositional differences among various source waters and between oil sands and commercial NAs; and, as a part of studies involving NAs toxicity to fish and mammals (Holowenko et al., 2002; Rogers et al., 2002a; Clemente et al., 2003a; Clemente et al., 2004; Clemente and Fedorak, 2005; Scott et al., 2005; Del Rio et al., 2006; Nero et al., 2006a; Biryukova et al., 2007; Han et al., 2008). For example, some of these differences or changes in the fingerprint were associated with the measured toxicity of OSPW. Using GC/MS Holowenko et al. (2002) identified an increase in the C22+ cluster, or the group of NAs with carbon numbers ≥22, which accompanied a decrease in the total NAs concentration and whole sample toxicity of OSPW. Moreover, the three dimensional bar graphs have been utilized to characterize NAs mixtures using analytical techniques other than GC/MS, such as ESI-MS (Rudzinski et al., 2002; Lo et al., 2003; Armstrong et al., 2008), APCI-MS (Lo et al., 2006), and electron impact high resolution MS (Tomczyk et al., 2001). Clemente et al. (2003a) successfully illustrated the application of a statistical comparison of NAs "fingerprints" produced by any MS procedure. Once the MS data are summarized in matrices a statistical test (e.g., *t*-test or analysis of variance) can be used to compare the distribution of ions or groups of ions that are different between various "fingerprints".

The common bench-top GC/MS instrument can be found in most analytical laboratories as opposed to the more specialized and relatively uncommon APCI and ESI mass spectrometry equipment (Clemente and Fedorak, 2005). The GC/MS technique provides the same information

for the characterization of NAs as the other more specialized MS techniques, but without the need for specialized reagent gases used in FI-MS as described by Dzidic et al. (1988), the need for electric fields as in the FAIMS method of Gabryelski and Froese (2003), or the need for specially trained operators (Holowenko et al., 2002).

However, the low resolution of the GC/MS method can be problematic. The potential misclassification of alcohols, natural fatty acids, and oxidation byproducts, along with the false positive detections that can occur using the low resolution GC/MS techniques can have important implications when trying to study NAs profile shifts and associated changes in toxicity. In recent years, more emphasis has been placed on using high resolution techniques to try to elucidate the structure-activity relationship of NAs, especially with respect to biodegradation and the ageing of OSPW (Bataineh et al., 2006; Merlin et al., 2007; Han et al., 2008; Han et al., 2009).

It has been demonstrated that the GC/MS method overestimates the relative proportions of low and high-molecular-mass acids (i.e., <C9 and/or >C22) when compared to newly available techniques with higher resolution (Clemente and Fedorak, 2005; Bataineh et al., 2006). Alcohols, natural fatty and organic acids may also erroneously contribute to false NAs detection and characterization. For instance, Merlin et al. (2007) used both low resolution and high resolution gas chromatography to compare NAs in surface and ground waters, and various samples from the petroleum industry. High resolution analysis was conducted using GC-HRMS with selected ion monitoring (SIM) mode after solid phase extraction (SPE), derivatization with t-BDMS, and subsequent bromination of the NA derivatives. Bromination and SIM were used to increase the specificity of this method. In a few cases, the GC-HRMS method with SIM indicated the absence of NAs, where the GC-LRMS indicated their presence. This was attributed to the increased specificity of the high resolution method and the possible misclassification of derivatized alcohols as NAs.

In the low resolution GC/MS method, Clemente et al. (2004) mentioned the possibility of NAs biotransformation products to be misclassified as high molecular weight NAs. A “double derivative” may be formed when a parent NA that undergoes microbial oxidation on the parent

ring or side chain forms a hydroxylated NA, which then reacts with the derivatizing agent yielding a di-(t-BDMS) derivative. Spectral analysis of this double derivative based on the *m*/*z* ratio would result in an incorrect assignment of the ion as a NA with a high carbon number instead of a NA with a lower carbon number. The misclassification of derivatives was confirmed by analyzing the same or similar samples used in the GC/MS studies to the new data collected using high resolution high performance liquid chromatography/quadrupole time of flight mass spectrometry (HPLC/QTOF-MS) (Bataineh et al., 2006). Oxidized intermediates can also undergo a second oxidation step that introduces a second reactive hydroxyl group (i.e. bi-hydroxylated NAs) (Bataineh et al., 2006). The presence of hydroxylated NAs was highlighted in chromatograms and can be considered a signature of NAs microbial transformation (Han et al., 2008). The biodegradation pathways for cycloalkyl carboxylic acids always lead to the production of these intermediates (Quagraine et al., 2005a; Han et al., 2008) making it difficult to characterize NAs mixtures using conventional mass spectrometry. In fact, there is a good possibility that the presence of the highly significant C22+ cluster, first highlighted by Holowenko et al. (2002) may be artefacts created by the double derivatization of hydroxylated NAs (Bataineh et al., 2006).

Around the same time, the use of high performance liquid chromatography (HPLC) in conjunction with high resolution mass spectrometry (HRMS) or quadrupole time-of-flight (QTOF) confirmed the presence of these misclassified NAs as oxidized NAs. In addition, a structure-activity relationship based on carbon number and *Z* series was identified as being a marker for biodegradation. Together, the data suggested that older water from reclamation ponds that had undergone extensive biodegradation of NAs as compared to the relatively fresh oil sands ore extract had lower Z_{-2} to Z_{-4}, Z_{-6}, and Z_{-8} ratios and contained more oxidized NAs (Han et al., 2009).

Common ionization techniques used in conjunction with mass spectrometry were unsuccessful at identifying peaks in the mass spectra that did not correspond to the general formula $C_nH_{2n+z}O_2$ (i.e., classical NAs). The use of instrumentation with higher resolving power and mass accuracy, such as atmospheric pressure photoionization (APPI) and electrospray ionization (ESI) with Fourier transform ion cyclotron resonance (FTICR) mass spectrometry, improved detection and

characterization of NAs mixtures (Barrow et al., 2010). Using both negative and positive ion modes, a greater number of peaks within the mass spectra of NAs mixtures could be observed. The negative ion mode was more amenable for acidic components and the positive ion mode was more suited for the characterization of basic components like sulfur adducts or other heteroatoms (Barrow et al., 2010). The end result was the identification of the presence of sole and oxidized sulfur and nitrogen containing species; oxy-naphthenic acids; and, naphthenoaromatics within complex oil sands NAs mixtures (Barrow et al., 2010). Unlike the FTIR method and low-resolution mass spectrometry, high- and ultra high-resolution mass spectrometric techniques can be used to differentiate humic and fulvic acids from NAs based on the degree of oxidation (i.e., humic and fulvic acids are more oxidized than NAs). This reduces the potential for interferences during the quantification and characterization of NAs, especially in complex water samples obtained from settling ponds and reclamation environments.

1.6 Salinity

The processing of oil sands not only leads to elevated levels of NAs, but also to increased salinity in produced waters. The principle ions that contribute to the increased salinity of OSPW are Na^+, Cl^-, SO_4^{2-}, HCO_3^{2-} and CO_3^{2-} (MacKinnon, 1989; MacKinnon et al., 2005). Increased salinity is largely from ions leaching from the ores, the process aids, and the geological formation in the case of ground water from deeper aquifers. The problem is further exacerbated by the recycling of water in the operating plants, which results in the production process waters with increasing ionic concentrations (Nero et al., 2006a). The total ionic content of OSPW can be greater than 2300 mg/L, but few studies have examined the interactive toxicity of NAs and salinity. Although naphthenates have been shown to adversely effect phytoplankton community structure, salinity has also been identified as a potential confounding factor (Leung et al., 2001). Nero et al. (2006a) examined the effects of salinity on NAs toxicity using local young-of-the-year yellow perch (*P. flavescens)*. Exposure to 25% of oil sands extracted NAs required to give an LC_{100}, resulted in high levels of gill proliferative changes. The toxic response was increased with the addition of 1 g/L Na_2SO_4. Moreover, a series of preliminary *C. dubia* tests conducted using artificial OSPW (reconstituted water with similar salt content, but free of the other constituents) gave a LC_{50} value similar to that obtained for the OSPW sample collected in the field (Chapter 3).

1.7 Reclamation at the Athabasca Oil Sands

Federal, provincial and municipal governments each have a role in the sustainable development of natural resources in Alberta, including resource development and environmental protection. The AOS development is no exception and is governed by environmental legislation and regulations. The primary piece of provincial legislation that provides for the protection of the environment is the *Environmental Protection and Enhancement Act* (*EPEA*). The *EPEA* governs the concept of site decommissioning and reclamation.

The definition of Reclamation according to the *EPEA* in the province of Alberta is:

> "the removal of equipment or buildings or other structures or appurtenances; the decontamination of buildings or other structures of appurtenances, or land or water; the stabilization, contouring, maintenance, conditioning or reconstruction of the surface of land; and any other procedure, operation or requirement specified in the regulations."

The definition of Land Reclamation is:

> "the reconditioning of the land to a state fit for some future use, and includes the stabilization, contouring, maintenance, conditioning, reconstruction and revegetation of the surface of land."

The objective of reclamation is to achieve maintenance-free, self-sustaining ecosystems (both aquatic and terrestrial) with capabilities equivalent to or better than pre-disturbance conditions (Golder Associates Ltd., 2000). Acceptable reclamation also includes post closure options (i.e., at the time of site abandonment) when intervention is not possible and control must be dependent on natural systems only (Bishay and Nix, 1996). The watercourses should be set on a path of ecological succession and should not require long-term maintenance and management.

The extraction and processing of oil sands has resulted in a number of environmental issues at the AOS including significant land disturbance, mobilization of chemicals associated with the bitumen, and generation of enormous volumes of tailings and fine tails. Section 23 of the *EPEA*

in Alberta prohibits the release of potentially toxic waste streams and requires that no oil sands tailings are deliberately released to ground or surface water supplies. As a result, process-affected waters and fluid tailings containing NAs and other organic and inorganic substances have been stored on-site primarily in large settling ponds (Quagraine et al., 2005b). The process affected waters created during both the operational phase and the post abandonment phase will likely require some level of treatment prior to its discharge into the natural environment due to the potential acute and chronic toxic effects to aquatic habitats (Bishay and Nix, 1996). As a consequence, containment areas must ultimately be reclaimed and potential long-term reclamation options for the tailings are actively being investigated.

1.7.1 Reclamation Strategies

Although there are other reclamation strategies governing solid waste and terrestrial disturbance (Golder Associates Ltd., 2000), the focus of this research is on liquid wastes or OSPW, which includes the tailings ponds and process-affected water. Reclamation of the tailings ponds water is considered to be the major environmental issue facing the oil sands industry. Together, government and industry formed the Oil Sands Water Release Technical Working Group (OSWRTWG) to highlight the work required to evaluate the acceptability of releasing OSPW into the environment. Specifically, this requires the removal of any acute or chronic toxicity from the water and achieving an effluent water chemistry that meets regulatory requirements (Quagraine et al., 2005b). No single technically, environmentally, and economically viable reclamation strategy has been developed in order to address both the volume of tailings produced and the associated toxicity, in particular the NAs and their potential to cause chronic toxicity to aquatic organisms (Quagraine et al., 2005b). Current investigations of reclamation techniques include dry and wet landscape approaches with an emphasis on bioremediation for detoxification.

The dry landscape approach involves dewatering or incorporating fine tails into a solid deposit which can be reclaimed as a viable land surface (Rogers, 2003). This also includes the dry tailings sand separated from the extraction process. The addition of chemical coagulants, such as gypsum or calcium sulfate, to the extraction tailings to produce consolidated tailings (CT) has been investigated (Golder Associates Ltd., 2000; Quagraine et al., 2005b). However, depending

on the extent of water removal, NAs contaminated process water may inevitably drain or leach from the reclaimed areas as seepage, which would then require further treatment (Quagraine et al., 2005b).

In the wet landscape approach, the tailings will retain their fluid character to allow for aquatic reclamation landscapes, such as lakes and wetlands (Leung et al., 2001). The two approaches for wet landscape reclamation are water capping and wetlands (Quagraine et al., 2005b). Water capping involves the disposal of fine tails under a layer of water of sufficient depth to isolate them from direct contact with the surrounding environment and allow for the development of a functioning lake ecosystem (MacKinnon, 1989; Rogers, 2003). The fine tails would form the lake sediments and over time, undergo densification while releasing the pore water and its toxic components into the capping layer (MacKinnon, 1989). Naphthenic acids would likely exist in the water column and in the sediments of these lakes, however, it is anticipated that within one or two years of capping, concentrations of NAs and other organic compounds in the surface water would be reduced by natural degradation below levels that would be acutely toxic to aquatic organisms (Quagraine et al., 2005b). An important feature of the capping ponds is the capping water/fine tails interface. This interface supports significant microbial and benthic invertebrate populations and the biological activity may possibly provide a barrier to the movement of organics, such as NAs, during densification (Herman et al., 1994a).

The role of wetlands in oil sands reclamation is generally to provide hydrological control, treatment of process affected waters, and habitat for wildlife and water fowl (MacKinnon et al., 2001). The OSPW directed into wetlands originates from tailings ponds, seepage from dykes and berms, and runoff. The main types of wetlands used for reclamation are constructed, altered, and opportunistic (MacKinnon et al., 2001). In the past, constructed wetlands have been shown to reduce the acute toxicity of OSPW, but results have not been definitive for altering the chronic toxicity associated with OSPW (Bishay and Nix, 1996; Pollet and Bendell-Young, 2000; Hersikorn and Smits, 2011). Wetland remediation could potentially be an acceptable reclamation option to regulatory officials if it is shown that the toxicity of OSPW can be reduced significantly without necessarily reducing the concentration of NAs as a whole to the appropriate levels. The final objective being that the water must be capable of supporting functioning

biological ecosystems (Quagraine et al., 2005b). For example, MacKinnon et al. (2005) documented that toxic components in process-affected water seepage, such as NAs and NH_4^+, are attenuated in the Lower Beaver Creek surface water system. Reductions in salinity, NAs, NH_4^+, and toxicity were also noted, suggesting the presence of natural bioremediation processes.

2.0 RESEARCH OBJECTIVES

Toxicity of OSPW is at least partially related to the complex mixture of dissolved organic acids commonly referred to as NAs. The connection between the degradation of specific NAs components and the loss of aquatic toxicity, along with the rate of change has not been thoroughly investigated thus far. The aim of this research was to evaluate the natural degradation of NAs and the associated reduction in aquatic toxicity of OSPW obtained from two different commercial operators at the AOS.

In this work, the parallel investigation of aquatic toxicity and NAs analyses performed on OSPW maintained in flow-through, simulated laboratory wetland microcosms, were used to correlate the reduction in toxicity of OSPW with changes in total NAs concentration and composition (i.e., NAs fingerprinting) over a 52-week test period. Experimental manipulations included two sources of OSPW (one from Syncrude Canada Ltd. and one from Suncor Energy Inc.), different hydraulic retention times (HRTs; 40 and 400 days), and increased nutrient availability (nitrate and phosphate). Once the selective biodegradation of NAs in OSPW was identified, additional experiments, using batch reactor vessels were conducted to evaluate the potential for NAs sorption onto the wetland sediments used in the original microcosm study. Moreover, off-line column and batch chromatography investigations were conducted in an attempt to separate and describe the aquatic toxicity associated with isolated groups of NAs homologues within a given mixture. The effectiveness of two different anion exchange resins and three co-polymers was assessed.

Additionally, during the initial stages of this research, it was noted that the NAs total concentration and composition, and initial toxicity of OSPW, were highly variable and dependent on the source, and that the toxicological effects observed with OSPW could not simply be associated with NAs concentrations without addressing the influence of salinity (e.g., Na^+, Cl^-, SO_4^{2-}, HCO_3^-). As a result, preliminary research involved indentifying suitable OSPW sources and a salt tolerant biological test organism. High total ionic content can interfere with the assessment of the aquatic toxicity of OSPW when conducting standard bioassays using

freshwater organisms that are sensitive to salinity, potentially confounding results and data interpretation.

To our knowledge, at the onset of this research (2005), synoptic NAs profiling and toxicity testing had not been performed to evaluate temporal changes in NAs chemistry and toxicity. For the first time, the structure-persistence relationship of NAs mixtures and the associated toxicity is described for OSPW in simulated wetland environments with continual OSPW and NAs recharge. One of the potential roles of wetlands in oil sands reclamation is to stimulate NAs biodegradation and to reduce the associated toxicity of OSPW to aquatic organisms. The identification of the toxic properties of OSPW, how their composition changes over time, and the rates of change under different conditions, should help identify the type of wetland conditions best suited for NAs degradation, and the time required for mitigation of the acute and chronic aquatic toxicity associated with OSPW.

To address the goals outlined above, the specific objectives of this research were to:

1) Describe the relative influence of naphthenic acids and salinity on the aquatic toxicity of OSPW, and identify a suitable salt tolerant biological test organism for the simulated wetland microcosm study.

2) Determine whether the natural degradation of NAs is influenced by OSPW type, hydraulic retention time, and nutrient availability in flow-through, simulated wetland environments (microcosms).

3) Describe and quantify the selective biodegradation of NAs congeners and to correlate the observed changes in total NAs concentration and composition (i.e., NAs fingerprinting) with the aquatic toxicity of OSPW.

4) Describe and quantify potential changes in total concentration and composition of NAs in OSPW due to abiotic processes such as sorption to the highly organic sediment used in the wetland microcosm study.

5) Isolate and separate an OSPW NAs mixture using off-line anion exchange chromatography and batch-wise co-polymer filtration and elution in order to determine the toxicological properties associated with specific groups of NAs homologues.

3.0 PRELIMNARY INVESTIGATIONS INTO DETERMINING THE CONTRIBUTIONS OF NAPHTHENIC ACIDS AND SALINTIY TO THE TOTAL AQUATIC TOXICITY OF OIL SANDS PROCESS-AFFECTED WATERS

3.1 Abstract

Oil sands process-affected waters (OSPW) at the Athabasca oil sands (AOS) contain a mixture of organic and inorganic constituents, including naphthenic acids (NAs) and salts. In the past, NAs have been shown to have inhibitory or toxic effects on a variety of organisms including plants, rodents, fish, snails, zooplankton, and bacteria. In this research, 7-day *Ceriodaphnia dubia* chronic toxicity tests using both un-manipulated (containing NAs) and manipulated (most NAs removed) samples of OSPW identified salinity (e.g., Na^+ and HCO_3^-) as a potential contributing factor to the overall aquatic toxicity of this complex water. Only a 5% reduction in acute toxicity and an 11% reduction in chronic toxicity was observed with a 91% reduction in total NAs concentration (from 67.2 mg/L to 5.9 mg/L; removed by solvent extraction). However, when the same samples were tested using the salt tolerant bacteria *Vibrio fischeri* in the Microtox® bioassay system, the acute and chronic toxicity was completely removed (EC_{50} and EC_{20} >100%). Additional *C. dubia* tests conducted using reconstituted water with similar salt composition and concentrations as in OSPW, but without any of the other potentially toxic constituents present (e.g., NAs), resulted in an LC_{50} of 1631 mg/L based on total ionic content. This LC_{50} value was similar to that obtained for the OSPW sample from the field (LC_{50} =1654 mg/L; back calculated to the total ionic content). Moreover, the LC_{50} for *C. dubia* in terms of HCO_3^- and Na^+ concentrations were estimated at 811 and 306 mg/L, respectively. The concentrations of HCO_3^- and Na^+ measured in OSPW were 940 and 574 mg/L, respectively. These constituents are present in greater concentrations than their respective LC_{50}s, thus contributing to the overall toxic effects of OSPW to the same aquatic organisms. Furthermore, an assessment of the mixture toxicity suggested a less than additive or antagonistic interaction between NAs and salts dissolved in OSPW. These results suggest that salts in OSPW may drive the toxicity of OSPW to the freshwater invertebrate *C. dubia*, and thus possibly other zooplankton species, and that the Microtox® bioassay may be better suited to track the overall toxic potential of NAs in OSPW.

3.2 Introduction

The Athabasca oil sands (AOS) is the largest of the four bitumen deposits located near Fort McMurray (56° 43' N, 111° 22' W) in northern Alberta, Canada (Barton and Wallace, 1979). An alkaline hot water extraction process is used to separate the surface mineable bitumen from the oil sands ore using large volumes of water (Schramm et al., 2000). This process results in the production of fluid tailings and oil sands process-affected waters (OSPW) containing, but not limited to, elevated levels of naphthenic acids and salinity. Due to an industry imposed zero discharge policy, and their associated toxic characteristics, fluid tailings and OSPW are stored on-site in designated settling basins that cover an area equalling an estimated 130 km^2 with a combined volume of approximately 10^9 m^3 (Han et al., 2009; Quesnel et al., 2011).

Contaminants present in OSPW, such as NAs and salts, are further concentrated when the overlying water contained in settling basins is recycled for reuse in the extraction process. As a result, NAs concentrations in OSPW can be as high as 120 mg/L (Clemente and Fedorak, 2005) and the total ion content may be greater than 2300 mg/L. Most of the aquatic toxicity of OSPW has been related to the persistent dissolved organic fraction of NAs (MacKinnon and Boerger, 1986; Headley and McMartin, 2004; Clemente and Fedorak, 2005). However, with in-situ half-lives on the order of 12.8 to 13.6 years associated with the more recalcitrant fractions of NAs (Han et al., 2009), toxicological effects observed with OSPW can not simply be associated with NAs concentrations without considering the influence of salinity. To date a few studies have examined the interactive toxicity of NAs and salinity on fish (van den Heuvel et al., 2000; Nero et al., 2006a) and phytoplankton community structure (Leung et al., 2001; Leung et al., 2003). The high salinity of process water can also impact terrestrial plants present near OSPW, especially in reclaimed areas. For example, survival of raspberry and strawberry seedlings was negatively affected in the presence of highly saline OSPW with a conductivity of 3000 μS/cm (Renault et al., 1998). Leaf necrosis was observed in conifer, strawberry, and raspberry seedlings, along with reduced transpiration rates and electrolyte leakage in strawberry and willow seedlings (Renault et al., 1998; Renault et al., 1999). These effects were attributed to the high salt content of the water, although the authors recognized that some of these effects could be attributed to the accumulation of other toxic components of the process water, and salt-induced nutritional deficiencies (Renault et al., 1998).

A few toxicological assays have been conducted on freshwater invertebrates using tailings waters from oil sands processing, but without a specific assessment of the contributions from elevated levels of salinity. MacKinnon and Boerger (1986) measured the toxicity of oil sands tailings pond water and found the LD_{50} for *Daphnia magna* was 2% v/v. Aquatic toxicity tests with *Hyalella azteca* and *D. magna* have shown that fresh tailings water is acutely toxic, as well as chronically toxic to *D. magna* after 1 to 2 years of ageing (Leung et al., 2001). In addition, *in-situ* and laboratory toxicity assays, using *D. magna* and *Ceriodaphnia dubia*, conducted with process waters introduced into constructed wetlands at the Suncor site showed some evidence of acute and chronic toxicity (Bishay and Nix, 1996). Overall, there is limited information for the combined effects of salinity and NAs on the aquatic toxicity of OSPW to freshwater invertebrates such as *C. dubia*.

Naphthenic acids composition and toxicity are source-specific and variability in test conditions can affect the bioavailability and toxicity of NAs to aquatic organisms (Rogers, 2003). It is important to note that in whole effluent toxicity studies using complex water samples (e.g., OSPW), the observed biological responses could potentially be a result of the exposure to other contaminants present in the test waters. Factors such as dissolved oxygen, temperature, salinity, and hardness, (CEATAG, 1998; Siwik et al., 2000) may increase or decrease the sensitivity of the biota to NAs, or cause toxicity themselves. Therefore, toxicity data and results from such tests should be interpreted with caution.

This preliminary investigation was part of a larger simulated wetland microcosm study that focused on the identification of the toxic properties of OSPW, the biodegradation of naphthenic acids under different environmental conditions, and the associated reduction in toxicity of OSPW to aquatic organisms. The results from this chapter were used to determine the contributions of salinity and naphthenic acids to the total aquatic toxicity of oil sands process-affected waters in order to identify a suitable test organism that would respond to NAs concentrations while tolerating the high ionic content of OSPW.

3.3 Materials and Methods

3.3.1 Test Waters

Oil sands process-affected water was collected from a dyke seepage location at the Mildred Lake Settling Basin (MLSB) on Syncrude Canada Ltd.'s (Syncrude) operational site at the AOS. The water was collected in May 2006 and transported to the Toxicology Centre, University of Saskatchewan, Saskatoon, SK, in plastic carboys. This OSPW (i.e., OSPW Dyke Seepage) was considered to be representative of the type of water that would be reclaimed in aquatic landscapes by industrial operators at the AOS and contained a total NAs concentration of 67.2 mg/L. A second sample of OSPW, referred to as NA-Low OSPW, was collected from a coke slurry discharge point on Syncrude's lease site in August 2006 and shipped to the Toxicology Centre in a plastic container. The NA-Low OSPW contained reduced concentrations of NAs (e.g., 5.9 mg/L NAs), but had a similar total ion content (i.e. salinity) to the original dyke seepage OSPW.

A sub-sample of the dyke seepage OSPW was solvent extracted to reduce the total NAs concentration (i.e., OSPW DCM-Extracted). Two litres of dyke seepage OSPW water was acidified to pH 2.5 using concentrated (18.6 M) H_2SO_4. Approximately 500 mL of the acidified OSPW was shaken for 2 min in a 1-L glass separatory funnel with 400 mL of reagent grade dichloromethane (DCM). The water and organic solvent phases were allowed to separate for 3 min before the more dense solvent phase was drained into a waste beaker. A second 200-mL aliquot of DCM was added to the remaining acidified OSPW and the process was repeated to ensure that any residual organic compounds were removed. This extraction procedure was repeated twice for a total of four times, until the entire 2-L of acidified OSPW had been solvent extracted, collected, and combined into a 6-L glass erlenmeyer flask. The 6-L flask containing the manipulated OSPW or OSPW DCM-Extracted (i.e., NAs and other organic compounds removed) was heated and stirred on a hot plate with a magnetic stirrer for 1.5 h with the temperature of the water maintained at 40°C. This step was performed to boil off any residual organic solvent that might have been left over in the manipulated OSPW sample from the liquid/liquid extraction step. The manipulated OSPW (i.e., OSPW DCM-Extracted) was then stirred magnetically overnight after which time the pH was re-adjusted to 7.92 using NaOH. The manipulation was repeated on 2-L of reconstituted water to be tested as the control in the

subsequent bioassays (i.e., Control DCM-Extracted). The reconstituted water used to prepare the Control DCM-Extracted sample was also used as dilution water in all *C. dubia* bioassays (i.e., Reconstituted Water). It had a pH between 7.8 and 8.5, and alkalinity and hardness ranging from 60 to 80 mg/L and 80 to 100 mg/L, respectively.

A test solution containing only oil sands NAs with minimal salinity was prepared by dissolving the NAs (6453 mg/L NAs) previously extracted and isolated from another sample of OSPW (Rogers, 2003) into reconstituted water. In order to achieve a similar total NAs concentration (64.5 mg/L) to that of the original dyke seepage OSPW (67.2 mg/L), 20 mL of this extract was dispensed into a 2-L glass volumetric flask containing reconstituted water (i.e., OSPW NAs in Recon. Water).

Artificial process water (i.e., Stock Salt Solution) was prepared to test salinity effects on *C. dubia* by dissolving salts into de-ionized water at similar ratios and concentrations to those present in the dyke seepage OSPW. The Stock Salt Solution was prepared using 400 mg/L NaCl, 700 mg/L Na_2SO_4, 3200 mg/L $NaHCO_3$, 200 mg/L $CaCl_2$, and 200 mg/L $MgCl_2$ to yield a total ionic content of 4700 mg/L. Thus, the concentration equal to 50% of the Stock Salt Solution was 2350 mg/L, which was reasonably similar to the total ionic content of the dyke seepage water (2032 mg/L). In addition, an individual salt test solution was prepared by dissolving $NaHCO_3$ into reconstituted dilution water to a final concentration of 6496 mg/L $NaHCO_3$ (i.e., $NaHCO_3$ Solution). The physical and chemical water quality characteristics of all test waters used in the *C. dubia* and Microtox® bioassays are summarized in Table 3.1.

Table 3.1. Physical and chemical properties (mean ± SD) of control and test waters used in the *Ceriodaphnia dubia* toxicity bioassays.

Water Quality Parameters		Recon. Water	OSPW Dyke Seepage	OSPW DCM-Extracted	Control DCM-Extracted
Temperature (°C)	(Initial)	23.8 ± 0.5 (46)	23.0 ± 1.2 (26)	23.2 ± 1.1 (30)	24.2 ± 0.9 (30)
	(Final)	24.6 ± 0.67 (137)	24.1 ± 0.2 (76)	24.2 ± 0.5 (85)	24.8 ± 0.2 (90)
Dissolved Oxygen (mg/L)	(Initial)	7.0 ± 0.4 (46)	7.6 ± 0.3 (27)	7.7 ± 0.4 (30)	7.2 ± 0.3 (25)
	(Final)	7.1 ± 0.3 (138)	7.4 ± 0.5 (75)	7.3 ± 0.5 (84)	6.9 ± 0.2 (90)
pH	(Initial)	8.1 ± 0.1 (46)	8.3 ± 0.1 (25)	7.7 ± 0.4 (30)	7.8 ± 0.3 (30)
	(Final)	8.3 ± 0.2 (46)	8.8 ± 0.2 (26)	7.7 ± 0.4 (30)	7.7 ± 0.3 (30)
Alkalinity (mg/L $CaCO_3$)		69 ± 9 (26)	782 (1)	18 ± 2 (4)	19 ± 4 (3)
Hardness (mg/L $CaCO_3$)		84 ± 5 (26)	90 (1)	93 ± 6 (4)	81 ± 3 (3)
Conductivity (µS/cm)		321 ± 11 (23)	2797 (1)	3840 ± 36 (3)	729 ± 8 (3)
Ammonia (mg/L-N)		n/a	2.30 ± 0.01 (2)	2.49 ± 0.11 (2)	< 0.01 (1)
Naphthenic Acids (mg/L)		n/a	67.2 (1)	5.9 (1)	< 0.5 (1)
Total Ionic Content (mg/L)		n/a	2032	n/a	n/a

n/a = not available.
Recon. = reconstituted.
NAs = naphthenic acids.
OSPW = oil sands process-affected waters.
DCM = dichloromethane.
Sample size (*n*) is presented in brackets.

Table 3.1 continued

Water Quality Parameters		OSPW NAs in Recon. Water	NA-Low OSPW	Stock Salt Solution	$NaHCO_3$ Solution
Temperature (°C)	(Initial)	24.1 ± 1.0 (35)	24.0 ± 0.5 (31)	23.7 ± 0.5 (21)	23.7 ± 0.5 (17)
	(Final)	24.0 ± 0.6 (102)	24.7 ± 0.3 (85)	24.4 ± 0.6 (53)	24.6 ± 0.5 (49)
Dissolved Oxygen (mg/L)	(Initial)	7.1 ± 0.4 (36)	7.7 ± 0.4 (26)	6.9 ± 0.4 (21)	7.4 ± 0.3 (17)
	(Final)	7.1 ± 0.3 (101)	7.3 ± 0.2 (85)	7.1 ± 0.7 (53)	7.2 ± 0.2 (49)
pH	(Initial)	8.0 ± 0.3 (35)	8.6 ± 0.1 (30)	8.4 ± 0.1 (21)	8.9 ± 0.3 (17)
	(Final)	8.3 ± 0.1 (35)	8.8 ± 0.2 (30)	9.0 ± 0.2 (20)	9.2 ± 0.1 (17)
Alkalinity (mg/L $CaCO_3$)		72 ± 2 (3)	481 ± 1 (2)	1640 (1)	3610 (1)
Hardness (mg/L $CaCO_3$)		77 ± 2 (3)	77 ± 1 (2)	266 (1)	98 (1)
Conductivity (µS/cm)		412 ± 5 (4)	3240 (1)	5240 (1)	5980 (1)
Ammonia (mg/L-N)		n/a	7.67 ± 0.20 (2)	n/a	< 0.01 (1)
Naphthenic Acids (mg/L)		64.5 (1)	6.9 (1)	n/a	n/a
Total Ionic Content (mg/L)		n/a	2196	4700	6496

n/a = not available.
Recon. = reconstituted.
NAs = naphthenic acids.
OSPW = oil sands process-affected waters.
DCM = dichloromethane.
Sample size (*n*) is presented in brackets.

3.3.2 Toxicity Assessment

Toxicological bioassays using *C. dubia* and the Microtox® system were conducted at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK. The freshwater invertebrate *C. dubia*, was used to assess the lethal and sub-lethal (fecundity) toxicity of all test waters in an environmental chamber maintained at 25 ± 1°C with a photoperiod of 16:8 hour light:dark. Culturing of the test organisms and performance of the chronic, three-brood, 7-d toxicity tests were conducted according to the Environment Canada (1992) test method. Acute and chronic toxicity tests were performed using *C. dubia* neonates that were cultured and maintained in moderately hard reconstituted water, ≤42 h old, and all born within 8 h of each other. For each test water, individual neonates were placed in 30-mL glass beakers containing 20 mL of a control or test water dilution series (e.g., 100%, 75%, 50%, 25%, and 10%) diluted with reconstituted water. Ten 30-mL beakers served as replicates for each of the control and sample dilutions. Test solutions were renewed every 24 h and test organisms in each 30-mL beaker were fed 0.1 mL of YCT (yeast, Cerophyll™, and trout chow) and 0.15 mL of algae (*Pseudokirchneriella subcapitata*) daily. The survival and number of offspring produced per replicate were recorded daily. The bioassay was considered to be a success if 80% of the controls had survived and 60% of the surviving adults had produced at least three broods with ≥15 neonates per adult.

The Microtox® bioassay is frequently used to monitor the toxicity of oil sands tailings water, and surrogate and commercial NAs mixtures. This bioassay uses the salt tolerant bioluminescent bacteria *Vibrio fischeri* (formerly *Photobacterium phosphoreum*) as the test organism, and the speed, relatively low cost, and reproducibility of the assay makes it ideal for testing the complex OSPW samples. The Microtox® M500 Toxicity Analyzer (Azur Environmental, formerly Microbics Corporation, Carlsbad, CA) and related computer software (Microtox® Ver 7.11) (Azur Environmental, 1989) were used to assess the toxicity of OSPW Dyke Seepage, OSPW DCM-Extracted (manipulated), Control DCM-Extracted, and OSPW NAs in Reconstituted Water samples. The 15 minute, 100% Basic Test was used to estimate the EC_{50} and EC_{20} of each sample under optimum conditions as described in the Microtox® manual, A Toxicity Testing Handbook (Azur Environmental, 1989). Furthermore, two reference toxicants, phenol and zinc sulfate, were analyzed to verify the performance of the Microtox® system. All pH adjustments and testing of reference toxicants and test samples were completed according to the

manufacturer's protocol (Azur Environmental, 1989). The measured phenol and zinc sulfate EC_{50} values were within their recommended ranges of 13 to 26 mg/L and 3 to 10 mg/L, respectively. Supplies for the Microtox® system were purchased from Osprey Scientific (Edmonton, AB).

3.3.3 Water Chemistry

Test water temperature, dissolved oxygen (DO) concentrations, and pH measurements were taken during the 7-d test period of the *C. Dubia* bioassay for all dilutions. Temperature and DO concentrations were measured using an Orion DO Meter Model 835 (Orion Research, Beverly, MA, USA) and an Oxi 315i DO Meter (WTW GmbH & Co, Weilheim, Germany). Sample water pH was measured an Orion Model 835 pH meter (Beverly, MA). The alkalinity, total hardness, conductivity, and ammonia measurements were conducted on the 100% sample (i.e., no dilution) of each test water. Alkalinity and hardness were measured using a HACH Digital Titrator Model 16900 (Hach Company, Loveland, CO). Conductivity was measured with an Orion Model 170 Conductivity Meter (Orion Research Inc., Boston, MA) and the total ammonia concentration, expressed as total ammonia-nitrogen, was determined using a SympHony pH/ISE Meter VWR Model SB301 (Thermo Fisher Corporation, Beverly, MA, USA). Total ion content was calculated by summing major ion concentrations determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Total NAs concentrations were measured using Fourier transform infrared (FTIR) spectroscopy according to methods described elsewhere (Jivraj et al., 1995; Holowenko et al., 2002). Both the major ion and NAs analyses were conducted by Syncrude Canada Ltd. (Edmonton, AB, Canada).

3.3.4 Statistical Analysis

For all of the *C. dubia* bioassays, the median lethal concentration (LC_{50}) estimates for survival, and the median inhibition concentration (IC_{50}) estimates for reproduction were calculated using the trimmed Spearman-Karber (TSK) method (version 1.5) (U.S. Environmental Protection Agency, 1990), and the Inhibition Concentration (ICp) approach (version 2.0) (U.S. Environmental Protection Agency, 1993), respectively. Microtox® EC_{50} estimates and 95% confidence intervals were obtained directly from the associated computer software (Microtox® Ver 7.11) (Azur Environmental, 1989).

3.3.5 Mixture Toxicity

The mixture toxicity of salts and NAs to *C. dubia* was assessed using Equation 3.1:

$$Am/Ai + Bm/Bi = S \quad \text{(Eqn. 3.1)}$$

where *A* and *B* are chemicals, *i* and *m* are the toxicities (LC_{50}s) of *A* and *B individually* and in a *mixture*, and *S* is the sum toxic action (Marking, 1985). This technique is based on the isobole theory and the toxic unit concept, and assumes that *A* and *B* are equitoxic and have similar modes of action (Marking, 1985). The sum *S*, can be used as a quantitative indicator of additive toxicity where *S* values greater than 1.0 suggest less than additive toxicity and *S* values less than 1 indicate greater than additive toxicity. To correct for the non-linearity of *S* values greater than and less than 1.0, and to assign *zero* as the reference point for simple additive toxicity, the additive index was calculated according to Equations 3.2 or 3.3. An additive index of *zero*, > *zero*, or < *zero*, suggests additive, greater than additive, and less than additive toxicity, respectively (Marking, 1985).

$$\text{Additive index} = 1/S - 1.0, \text{ for } S \leq 1.0 \quad \text{(Eqn. 3.2)}$$

$$\text{Additive index} = S(-1) + 1, \text{ for } S \geq 1.0 \quad \text{(Eqn. 3.3)}$$

3.4 Results

3.4.1 Aquatic Toxicity of Test Waters

A comparison between the un-manipulated OSPW Dyke Seepage and the OSPW DCM-Extracted (manipulated) water samples using *C. dubia* showed that some of the initial toxicity remained after extraction, even with a reduction of NAs from an initial concentration of 67.2 to 5.9 mg/L (Table 3.2). There was only a 5% reduction in acute toxicity (initial LC_{50} increasing from 81.4 to 86.4%) although there was a 91% reduction in total NAs concentration. Similarly, only an 11% reduction in chronic toxicity was observed (initial EC_{50} was improved from 65.3 to 76.3%) with the reduction in NAs. The possibility that the solvent extraction step itself contributed to the residual toxicity was ruled out, because the LC_{50} and EC_{50} of the Control DCM-Extracted water was >100%. The LC_{50} and EC_{50} of the solution containing the extracted OSPW NAs re-dissolved in Reconstituted Water was equivalent to 39.4 and 8.8 mg/L NAs,

respectively, and the LC_{50} and EC_{50} of NA-Low OSPW was estimated at 3.0 and 1.4 mg/L, respectively (Table 3.2). The toxicity of the NA-Low OSPW that had a total NAs concentration of 5.9 mg/L was much lower (i.e., more toxic) than that of the pure OSPW NAs re-dissolved in Reconstituted Water.

Table 3.2. Summary of the *Ceriodaphnia dubia* bioassay results (lethal and sublethal) for various test waters.

Acute and Chronic Toxicity	OSPW Dyke Seepage	OSPW DCM-Extracted	Control DCM-Extracted	OSPW NAs in Recon. Water
Survival - LC50 (% v/v)	81.4	86.4	> 100	61.2
95% C.I. (upper - lower)	(90.3 - 73.4)	(99.2 - 75.3)	n/a	(80.8 - 46.4)
Reproduction - EC50 (% v/v)	65.3	76.3	> 100	13.7
95% C.I. (upper - lower)	(69.9 - 54.7)	(80.2 - 69.8)	n/a	(17.2 - 11.4)
Survival - LC50 (mg/L NAs)	54.7	5.1	n/a	39.4
95% C.I. (upper - lower)	(60.7 - 49.3)	(5.9 - 4.4)	n/a	(52.1 - 29.9)
Reproduction - EC50 (mg/L NAs)	43.8	4.5	n/a	8.8
95% C.I. (upper - lower)	(47.0 - 36.8)	(4.7 - 4.1)	n/a	(11.1 - 7.4)
Survival - LC50 (mg/L total ionic content)	1654	n/a	n/a	n/a
95% C.I. (upper - lower)	(1835 - 1491)	n/a	n/a	n/a
Reproduction - EC50 (mg/L total ionic content)	1326	n/a	n/a	n/a
95% C.I. (upper - lower)	(1420 - 1112)	n/a	n/a	n/a
Survival - LC50 (mg/L Na^+)	n/a	n/a	n/a	n/a
95% C.I. (upper - lower)	n/a	n/a	n/a	n/a
Reproduction - EC50 (mg/L Na^+)	n/a	n/a	n/a	n/a
95% C.I. (upper - lower)	n/a	n/a	n/a	n/a
Survival - LC50 (mg/L HCO_3^-)	n/a	n/a	n/a	n/a
95% C.I. (upper - lower)	n/a	n/a	n/a	n/a
Reproduction - EC50 (mg/L HCO_3^-)	n/a	n/a	n/a	n/a
95% C.I. (upper - lower)	n/a	n/a	n/a	n/a

n/a = not available.
Recon. = reconstituted.
NAs = naphthenic acids.
OSPW = oil sands process-affected waters.
DCM = dichloromethane.
LC = lethal concentration.
EC = effective concentration.
C.I. = confidence interval.

Table 3.2 continued

Acute and Chronic Toxicity	NA-Low OSPW	Stock Salt Solution	$NaHCO_3$ Solution
Survival - LC50 (% v/v)	42.9	34.7	17.2
95% C.I. (upper - lower)	(54.1 - 34.1)	n/a	(20.1 - 14.7)
Reproduction - EC50 (% v/v)	20.0	24.8	13.2
95% C.I. (upper - lower)	(23.2 - 16.9)	(30.2 - 19.4)	(16.1 - 9.3)
Survival - LC50 (mg/L NAs)	3.0	n/a	n/a
95% C.I. (upper - lower)	(3.7 - 2.4)	n/a	n/a
Reproduction - EC50 (mg/L NAs)	1.4	n/a	n/a
95% C.I. (upper - lower)	(1.6 - 1.2)	n/a	n/a
Survival - LC50 (mg/L total ionic content)	942	1631	1117
95% C.I. (upper - lower)	(1188 - 749)	n/a	(1306 - 955)
Reproduction - EC50 (mg/L total ionic content)	439	1166	857
95% C.I. (upper - lower)	(509 - 371)	(1419 - 912)	(1046 - 604)
Survival - LC50 (mg/L Na^+)	n/a	n/a	306
95% C.I. (upper - lower)	n/a	n/a	(358 - 262)
Reproduction - EC50 (mg/L Na^+)	n/a	n/a	235
95% C.I. (upper - lower)	n/a	n/a	(286 - 165)
Survival - LC50 (mg/L HCO_3^-)	n/a	n/a	811
95% C.I. (upper - lower)	n/a	n/a	(948 - 694)
Reproduction - EC50 (mg/L HCO_3^-)	n/a	n/a	623
95% C.I. (upper - lower)	n/a	n/a	(760 - 439)

n/a = not available.
Recon. = reconstituted.
NAs = naphthenic acids.
OSPW = oil sands process-affected waters.
DCM = dichloromethane.
LC = lethal concentration.
EC = effective concentration.
C.I. = confidence interval.

The total ionic content of OSPW Dyke Seepage that resulted in the 50% mortality in *C. dubia* (LC_{50} =1654 mg/L) was similar to the LC_{50} for the Stock Salt Solution (LC_{50} =1632 mg/L) (Table 3.2). Furthermore, the LC_{50} for *C. dubia* in terms of HCO_3^- and Na^+ concentrations were estimated at 811 and 306 mg/L, respectively (Table 3.2), which is within the range of the typical concentrations found in dyke seepage OSPW. The HCO_3^- and Na^+ concentrations in OSPW Dyke Seepage used in this study were 940 and 574 mg/L, respectively (data not shown). Collectively, the bioassays conducted using the Stock Salt and the $NaHCO_3$ Solutions suggest that the observed *C.dubia* toxicity may be the result of the total ionic content and more specifically the HCO_3^- and Na^+ ions.

Vibrio fishceri, the bacterial test organism used in the Microtox® bioassays, were more sensitive (EC_{50} and EC_{20} equal to 57.8% and 15.7%, respectively) to the OSPW Dyke Seepage sample than *C. dubia*. Moreover, the acute and chronic toxicity of OSPW Dyke Seepage was completely removed (EC_{50} and EC_{20} >100%) after the NAs were largely removed by DCM extraction. Similar to the *C.dubia* bioassay, the Control DCM-Extracted water sample was not toxic (EC_{50} >100%) (Table 3.3).

Table 3.3. Summary of Microtox® bioassay results (EC_{50} and EC_{20}) for various test waters.

Acute and Chronic Toxicity	OSPW Dyke Seepage	OSPW DCM-Extracted	Control DCM-Extracted
Acute toxicity - EC50 (% v/v)	57.8	> 100	> 100
95% C.I. (upper - lower)	(66.5 - 50.3)	n/a	n/a
Chronic toxicity - EC20 (% v/v)	15.7	> 100	> 100
95% C.I. (upper - lower)	(13.0 - 19.0)	n/a	n/a
Acute toxicity - EC50 (mg/L NAs)	38.8	> 100	> 100
95% C.I. (upper - lower)	(44.7 - 33.8)	n/a	n/a
Chronic toxicity - EC20 (mg/L NAs)	10.6	> 100	> 100
95% C.I. (upper - lower)	(8.7 - 12.8)	n/a	n/a
Acute toxicity - EC50 (mg/L total ionic content)	1174	> 100	> 100
95% C.I. (upper - lower)	(1351 - 1022)	n/a	n/a
Chronic toxicity - EC20 (mg/L total ionic content)	319	> 100	> 100
95% C.I. (upper - lower)	(264 - 386)	n/a	n/a

n/a = not available.
NAs = naphthenic acids.
OSPW = oil sands process-affected waters.
DCM = dichloromethane.
EC = effective concentration.
C.I. = confidence interval.

3.4.2 Mixture Toxicity of Naphthenic Acids and Dissolved Salts

The interactive toxicity of NAs and salinity to *C.dubia* was calculated using the LC_{50}s of the different test waters assessed in the previous bioassays (Table 3.2). *Am* represented the *C. dubia* LC_{50} for dissolved NAs in OSPW Dyke Seepage. This OSPW contained both dissolved NAs and salts having a combined LC_{50} of 81.4% v/v. The LC_{50} for the NAs in this mixture was back calculated as 54.7 mg/L. The term *Ai* was represented by the *C. dubia* LC_{50} of OSPW NAs re-dissolved in Reconstituted Water (39.4 mg/L). *Bm* represented the *C. dubia* LC_{50} for total ionic content in OSPW Dyke Seepage. As before, the LC_{50} for total ionic content in this OSPW was back calculated as 1654 mg/L. The term *Bi* was the *C. dubia* LC_{50} of the Stock Salt Solution (i.e., artificial process water that did not contain any NAs) (1631 mg/L). The calculated *S* value, and the additive index for NAs and salt interaction was 2.40 and −1.40, respectively. Both the *S* value and the additive index suggest a less than additive toxicity or antagonistic effect.

3.5 Discussion

During the hot water extraction process utilized at the AOS, ions leached from the bitumen ore and those added by process chemicals increase the total ionic content of the process water (Renault et al., 1998). Increased levels of salinity in some OSPW is a challenge for reclamation, as biological and other more conventional treatment strategies are incapable of effectively removing dissolved salts from the large volumes of process-affected waters stored on-site. Not only are the toxic dissolved organic compounds a concern for stakeholders, but there is potential for the highly saline process waters to have environmental impacts on a variety of organisms that inhabit the regional freshwater ecosystems.

Research involving *C. dubia*, or other daphnids, and OSPW is sparse, and the few studies that do exist have not described or provided any data for the possibility of direct salt effects or for interactions between dissolved salts and NAs (MacKinnon and Boerger, 1986; Nix and Martin, 1992; Bishay and Nix, 1996). Leung et al. (2001) and (2003) demonstrated that along with NAs, the salinity in process waters could influence phytoplankton community structure in water bodies near oil sands operations with varying degrees of process water influence, age, and size. It was suggested that the ecological effect of major ions (100 to 3000 mg/L total dissolved ions) appeared to be at least as great as that of naphthenates, and also that the influence of salinity requires further investigation. The results from the *C. dubia* bioassays performed here involving DCM extraction of NAs from OSPW suggested that something other than NAs was responsible for residual OSPW toxicity and thus would contribute to the overall toxic effect of OSPW. It was shown that salinity (high total ionic content) was responsible for some of the observed toxicity of OSPW to *C. dubia*, as the bioassay conducted on the Stock Salt Solution yielded an LC_{50} which was nearly identical to the LC_{50} of the OSPW Dyke Seepage sample, based on the total ionic content. More specifically, the investigation using the $NaHCO_3$ Solution revealed that high concentrations of both the Na^+ cations and HCO_3^- anions could result in acute and chronic effects to *C. dubia*. Sodium and bicarbonate are the predominant ions in process waters where they can account for up to 95% of the cation, and 75% of the anion equivalents, respectively (MacKinnon, 1989). These two ions were present in concentrations that were within the range of the LC_{50}s for *C. dubia* estimated using the salt solutions prepared in this research.

In addition to the dissolved salts, some of the observed toxicity could be due to the other constituents, which if present in the complex OSPW samples at high enough concentrations, are know to cause toxicity to *C. dubia* and other freshwater organisms. For example, total ammonia concentrations in OSPW Dyke Seepage, OSPW DCM-Extracted, and NA-Low OSPW samples were 2.30, 2.49, and 7.67 mg/L-N, respectively (Table 3.1). Ammonia is highly water soluble and its speciation is affected by pH, temperature, and ionic strength (Canadian Council of Ministers of the Environment, 2010). Ammonia exists in both un-ionized (NH_3) and ionized (NH_4^+) forms, however, the toxicity to aquatic organisms is mostly linked to the un-ionized form and less frequently to total ammonia or the un-ionized ammonium ion (Andersen and Buckley, 1998). The Canadian Water Quality Guidelines for the Protection of Aquatic Life for total ammonia is 0.354 mg/L as NH_3 at pH 8 and 25°C (Canadian Council of Ministers of the Environment, 2010). Multiplying this value by a factor of 0.8224 converts this guideline value to 0.291 mg/L-N. Similarly, the recent US Environmental Protection Agency (US EPA) 1999 draft update for Ambient Water Quality Criteria for Ammonia in freshwater systems proposes an acute and chronic criterion of 2.9 and 0.26 mg/L-N, respectively (U.S. Environmental Protection Agency, 2009). More specifically, the LC_{50} of ammonia to *C. dubia*, for similar temperature (approximately 25°C) and pH (approximately 8) conditions to this research are reported to be between 1.73 to 1.00 mg/L-NH_3, or 1.42 to 0.822 mg/L-N (Arthur et al., 1987; Nimmo et al., 1989; Cowgill and Milazzo, 1991; Andersen and Buckley, 1998). The observed ammonia concentrations in this research were at or above the Canadian and U.S ambient freshwater quality guidelines and the LC_{50} estimates specific for *C. dubia*. As a consequence, the potential for ammonia contributing to the overall toxic effects could not be discounted.

The Microtox® acute toxicity data are presented as an EC_{50}, but in reality it is the inhibition of light production, which is a normal by product of *V. fishceri* respiration and metabolic processes (Azur Environmental, 1989), that is used to calculate the inhibition concentration (IC). The Microtox® assay does not produce quantal data, but rather is a measure of light loss or rate of biological activity (Azur Environmental, 1989). Thus, a measure for reproduction can not be obtained and the EC_{20} value was used as a surrogate for chronic toxicity. Mackinnon and Boerger (1986) have also used the Microtox® EC_{20} as a surrogate measure of chronic toxicity when analyzing OSPW and determined oil sands tailings water, using *V. fischeri*, to have an IC_{50}

between 20% and 30% (v/v) and an IC_{20} of 10% (v/v). Similarly, Herman et al. (1994a) and (1994b) concluded that naphthenic acid sodium salts have an EC_{50} value of 30% v/v. The slightly higher Microtox® EC_{50} value of OSPW Dyke Seepage (57.8% v/v) in this study was likely the result of differences in the source of the OSPW tested and potential differences in NAs concentrations and composition due to natural degradation. The Microtox® EC_{20} of OSPW Dyke Seepage (15.7% v/v) was more consistent with the previously reported value of 10% (v/v). The EC_{20} provides a 2.5-fold increase in sensitivity over the EC_{50} (Azur Environmental, 1989). Furthermore, the difference between the lower EC_{20} value (initial toxicity) and an EC_{20} >100% (lack of toxicity), presents a larger range of values over which toxicological recovery can be monitored.

The influence of salinity on *C. dubia* was further substantiated when the DCM extracted samples were tested using the Microtox® system. In contrast to the *C. dubia* bioassay, the 91% reduction of NAs in the OSPW DCM-Extracted sample completely removed the acute and chronic toxicity when measured using the salt tolerant *V. fischeri*. Therefore, the Microtox® toxicity better correlates with a reduction in total NAs concentrations and appears to be less influenced by salinity and other dissolved constituents present in OSPW (including ammonia) than the *C. dubia* bioassay.

The model described by Marking (1985) was used to determine if the toxicity of key OSPW constituents (salts and NAs) was additive, greater than additive, or less than additive. It was assumed that the observed toxicity of OSPW was not influenced by anything other than salts and NAs, even though the equation could have been expanded to include additional potentially toxic constituents of OSPW (e.g., ammonia). However, in order to simplify the initial assessment only the combined effects of NAs and salts were considered. The model also assumes that the toxic mode of action of the chemicals in question are the same (Marking, 1985). Naphthenic acids toxicity is not yet well understood, but narcosis has been suggested as the primary mode of action (Frank et al., 2008; Frank et al., 2010). This non-specific form of toxicity can result in the alteration of proper membrane functioning leading to the disruption of ion homeostasis and osmotic stress. Likewise, *C. dubia* and other freshwater organisms that are exposed to high levels of dissolved salts, would have a difficult time with salt regulation potentially leading to

internal ion and osmotic imbalances. Therefore, although the mode of actions are likely not identical, there are enough similarities to justify the use of this model.

In order to determine the significance of the calculated less than additive mixture toxicity effect, *S* values outside the range of 0.5 to 2.0 are considered to be significantly different from simple additive toxicity (Marking, 1985). The calculated *S* value for NAs and salinity in this research was 2.4, providing a reasonable level of confidence that the less than additive toxicity was significant. According to the definition of less than additive toxicity, the combination of NAs and salts in the same water sample should result in a higher LC_{50} (i.e., less toxic) than what would be expected for NAs alone. The results of the *C. dubia* bioassays are consistent with this definition as the LC_{50} of the extracted OSPW NAs re-dissolved in Reconstituted Water was lower than the LC_{50} of OSPW Dyke Seepage that contained both NAs and salts in combination. The calculated less than additive toxicity of NAs and salts is consistent with the results of Nero et al. (2006a) who showed yellow perch (*Perca flavescens*) exposed to NAs in combination with 1g/L Na_2SO_4, resulted in decreased mortality compared to those exposed to NAs alone. In contrast however, it was noted that gill proliferative alterations such as chloride, epithelial, and mucous cell proliferations were increased with exposure to NAs, at concentrations equivalent to 25% of the lethal dose (LC_{100}), in combination with 1 g/L Na_2SO_4. This inconsistency was attributed to differences in the relative composition of the NAs mixtures that were tested.

To obtain an accurate assessment of mixture toxicity, the model should be applied to the toxicity of the individual salts and not simply the total ionic content since certain salts are expected to have different toxicological threshold concentrations. More importantly, the use of this model should be further refined to include those NAs homologues, or groups of NAs homologues, that are responsible for the acute toxicity as separate chemicals in the mixture toxicity assessment equation. Athabasca oil sands NAs mixtures are made up of numerous closely related congeners with various physical, chemical and toxicological properties (Headley and McMartin, 2004). Therefore, the overall toxicological properties of a given NAs mixture is a function of the content and complexity of that given mixture as whole. The complexity or distribution of NAs congeners can also vary from source to source and change over time, especially within aquatic reclamation environments. Thus, any mixture assessment will be specific to the source of the

sample and its constituents, and extrapolation to other sources should be made with caution. To date, the complete separation of a given NAs mixture and subsequent toxicity testing of the separated fractions to determine the toxicity of individual NAs or groups of NAs homologues is an area of research that has eluded scientists. A few studies have tackled this challenge with limited success (Lo et al., 2006; Frank et al., 2008), however its importance to the accurate characterization of NAs toxicity remains paramount.

3.6 Conclusions

Salts present in OSPW appear to influence and may largely drive the toxicity of OSPW to the freshwater invertebrate *C. dubia*, and certain salts may be more of a concern than others. On the other hand, the Microtox® assay appears to respond largely to NAs and may thus be a more useful tool to track NAs toxicity with little interference from the other potentially toxic constituents in OSPW. As a result, the Microtox® bioassay was chosen to monitor the changes in OSPW toxicity associated with changes in NAs concentrations and complexity in the subsequently discussed simulated wetland experiments (Chapters 4 and 5). However, to identify the overall toxic potential of OSPW to freshwater organisms, the sensitivity of *C. dubia* provides a more accurate representation of the acute and toxic effects from all of the constituents present in OSPW. If data are not interpreted and used appropriately, the difference in toxic response between *C. dubia* and Microtox® could complicate the regulatory assessment of reclamation success, and the establishment of potential water quality criteria and guidelines for the eventual release of OSPW.

4.0 DEGRADATION AND AQUATIC TOXICITY OF NAPHTHENIC ACIDS IN OIL SANDS PROCESS-AFFECTED WATERS USING SIMULATED WETLANDS

This chapter has been submitted with minor modification for publication in the journal Chemosphere. The full citation is: Toor, N.S., Franz, E.D., Fedorak, P.M., MacKinnon, M.D., Liber, K. Degradation and aquatic toxicity of naphthenic acids in oil sands process-affected waters using simulated wetlands. Chemosphere (submitted).

4.1 Abstract

Oil sands process-affected waters (OSPW) produced during the extraction of bitumen at the Athabasca Oil Sands (AOS) located in northeastern Alberta, Canada, are toxic to many aquatic organisms. Much of this toxicity is related to a group of dissolved organic acids known as naphthenic acids (NAs). Naphthenic acids are a natural component of bitumen and are released into process water during the separation of bitumen from the oil sand ore by a caustic hot water extraction process. Using laboratory microcosms as an analogue of a proposed constructed wetland reclamation strategy for OSPW, we evaluated the effectiveness of these microcosms in degrading NAs and reducing the aquatic toxicity of OSPW over a 52-week test period. Experimental manipulations included two sources of OSPW (one from Syncrude Canada Ltd. and one from Suncor Energy Inc.), two different hydraulic retention times (HRTs; 40 and 400 days), and increased nutrient availability (added nitrate and phosphate). Microcosms with a longer HRT (for both OSPWs) showed higher reductions in total NAs concentrations (64 to 74% NAs reduction, P <0.05) over the test period, while nutrient enrichment appeared to have little effect. A 96-hour static acute rainbow trout (*Oncorhynchus mykiss*) bioassay showed that the initial acute toxicity of Syncrude OSPW (LC_{50} =67% v/v) was reduced (LC_{50} >100% v/v) independent of HRT. However, EC_{20}s from separate Microtox® bioassays were relatively unchanged when comparing the input and microcosm waters at both HRTs over the 52-week study period (P >0.05), indicating that some sub-lethal toxicity persisted under these experimental conditions. The present study demonstrated that given sufficiently long HRTs, simulated wetland microcosms containing OSPW significantly reduced total NAs concentrations and acute toxicity, but left behind a persistent component of the NAs mixture that appeared to be associated with residual chronic toxicity.

4.2 Introduction

At the Athabasca Oil Sands (AOS) in northeastern Alberta, Canada, the caustic hot water extraction process described by Clark in 1932 is still used by some companies with surface mining operations to separate bitumen from oil sand ore (Clark and Pasternack, 1932). With this aqueous method, large volumes of water are required during the extraction of bitumen, and during its upgrading to light sweet oil. In current integrated surface mining operations at the AOS, approximately 2 to 2.5 m^3 of fresh water is required to produce 1 m^3 of synthetic crude oil (W. Zubot, Syncrude Canada Ltd., Edmonton, AB, Canada, personal communication). At production rates that are approaching one million barrels of oil per day, this results in the build-up of large inventories of liquid tailings, of which oil sands process-affected waters (OSPW) is a major component and a significant stakeholder concern (MacKinnon, 1989; Holroyd and Simieritsch, 2009). Previous toxicological investigations have shown that freshly produced OSPW, and OSPW contained within the active tailings system, are toxic to various aquatic organisms (MacKinnon and Boerger, 1986; Leung et al., 2001; Nero et al., 2006b). The aquatic toxicity of OSPW has largely been linked to elevated concentrations of a relatively persistent group of dissolved organic acids known as naphthenic acids (NAs) (MacKinnon, 1989; Verbeek, 1994; Holowenko et al., 2002; Headley and McMartin, 2004). Currently, waters containing OSPW are not released from the oil sands mining lease sites into the natural water bodies of the region (Quagraine et al., 2005b). As a result, tailings and their OSPW are retained within large holding ponds and settling basins, but with the understanding that eventual reclamation of both of these materials must be undertaken. As of 2007, greater than one billion m^3 of OSPW, as water held within various tailings deposits, was stored within the AOS by Syncrude Canada Ltd. alone (Zubot, 2010). Successful reclamation of OSPW will require a reduction in NAs concentrations in the OSPW and the removal of the water's toxic character. Natural or enhanced bioremediation in lakes and wetlands within the lease closure landscapes will likely play a critical role in meeting these requirements. In this chapter, aspects relevant to the design and management of constructed or opportunistic wetlands to meet this goal are described.

Naphthenic acids are natural carboxylic acid components of the bitumen found in the oil sands of the McMurray AOS formation (Rogers, 2003). This is not unique, as NAs are found in most crude oils at concentrations varying between 0 to 4% (w/w), depending on the geographical

location of the petroleum reserve (Dzidic et al., 1988). Their levels have been reported to be about 0.1 to 2% (w/w) in the AOS (AOSTRA, 1989). During the bitumen extraction process, NAs are solubilised as carboxylates during the digestion of oil sands ore with alkaline hot water. Sodium hydroxide (NaOH) is used as a process aid and while dosages vary, the pH of the process water generally ranges from 8 to >10. Under these alkaline conditions NAs, which have a pK_a of ~5, will ionize and be solubilised into the extraction waters where they are present as sodium naphthenates in their dissociated form (Schramm et al., 2000; Leung et al., 2001; Clemente and Fedorak, 2005). Naphthenic acids become concentrated in the tailings pond water over time because OSPW from the tailings ponds is recycled repeatedly for reuse in the extraction process (Quagraine et al., 2005b). Oil sands process-affected waters typically contains total NAs concentrations ranging from 20 to 120 mg/L (Clemente and Fedorak, 2005) and generally do not exceed these levels due to the occurrence of some natural degradation.

Field observations at the AOS suggest that NAs in OSPW tailings ponds degrade very slowly (low oxygen environments), but even under aerobic conditions isolated from active tailings, the observed degradation of NAs is slow and incomplete (Holowenko et al., 2002). Complete microbial mineralization under natural conditions does not appear to be occurring and a portion of the NAs mixture, particularly the higher molecular weight NAs, appears to be resistant to further degradation (Quagraine et al., 2005a). Even after a period of 7 to 11 years, with no subsequent input of tailings material, the total concentration of NAs in experimental ponds containing fresh OSPW (~50 mg/L NAs) did not decrease below 20 mg/L (Leung et al., 2001; Holowenko et al., 2002). However, Herman et al. (1994b) demonstrated that mineralization of oil sands NAs, via microbial activity, was possible and that it corresponded to a reduction in Microtox® toxicity. Some residual (i.e., chronic) toxicity persisted, suggesting that this toxicity could be related to the more persistent NAs. However, a clear relationship between the persistent fraction of NAs and the associated persistent aquatic toxicity of OSPW has not been established and requires further investigation.

More recent studies have better demonstrated the pathways of degradation of OSPW NAs and the limitations of natural processes (Han et al., 2009). With improvements in analytical capabilities, better information on the refractory fractions of NAs has become available

suggesting that these refractory constituents are more associated with the presence of hydroxylated metabolites of degradation than with molecular weight and ring structures alone. The pathway(s) of natural degradation still needs to be fully understood as options for reclamation are actively being developed and deployed.

Using small simulated wetlands (laboratory microcosms), we evaluated the potential for NAs degradation, the influence of hydraulic retention time (HRT) and nutrient enrichment on NAs degradation, and the associated reduction in aquatic toxicity of OSPW generated by Syncrude Canada Ltd. (Syncrude) and Suncor Energy Inc. (Suncor). Laboratory microcosms, under flow-through conditions, were used to mimic wetland environments. Reclamation landscapes using both lakes and wetlands offer a strategy for the reclamation of OSPW at the AOS. A greater understanding of possible design and management criteria to optimize such aquatic environments for NAs remediation, and the associated rates of OSPW detoxification (e.g., thresholds for naphthenic acids toxicity to aquatic biota), will increase stakeholder confidence in performance goals within reclamation plans.

4.3 Materials and Methods

4.3.1 Experimental Design

Two sources of OSPW (Syncrude and Suncor) were used to charge and recharge flow-through, simulated wetland microcosms in the laboratory. Two exposure times were used to create wetland systems with a short (40-day) and long (400-day) hydraulic retention time (HRT). Nutrient levels were also modified by the addition of mineral forms of nitrogen ($NaNO_3$) and phosphorus (Na_2HPO_4) to provide a low (no added nutrients) and high (~1 mg/L N and ~150 µg/L P) nutrient loading. The resulting matrix of test conditions allowed for the evaluation of basic factors possibly linked to enhanced biodegradation of NAs and associated reduction in aquatic toxicity. Laboratory microcosms were constructed to simulate a simplistic wetland habitat similar to those that could be used in the reclamation of OSPW at the AOS. An array of indigenous microbial communities present in the input OSPW and in the sediments from a local wetland (both added to the microcosms) were the main sources of microbial activity in each system. Overall there were 12 different treatment scenarios, tested in triplicate, resulting in a total of 36 microcosms (Figure 4.1).

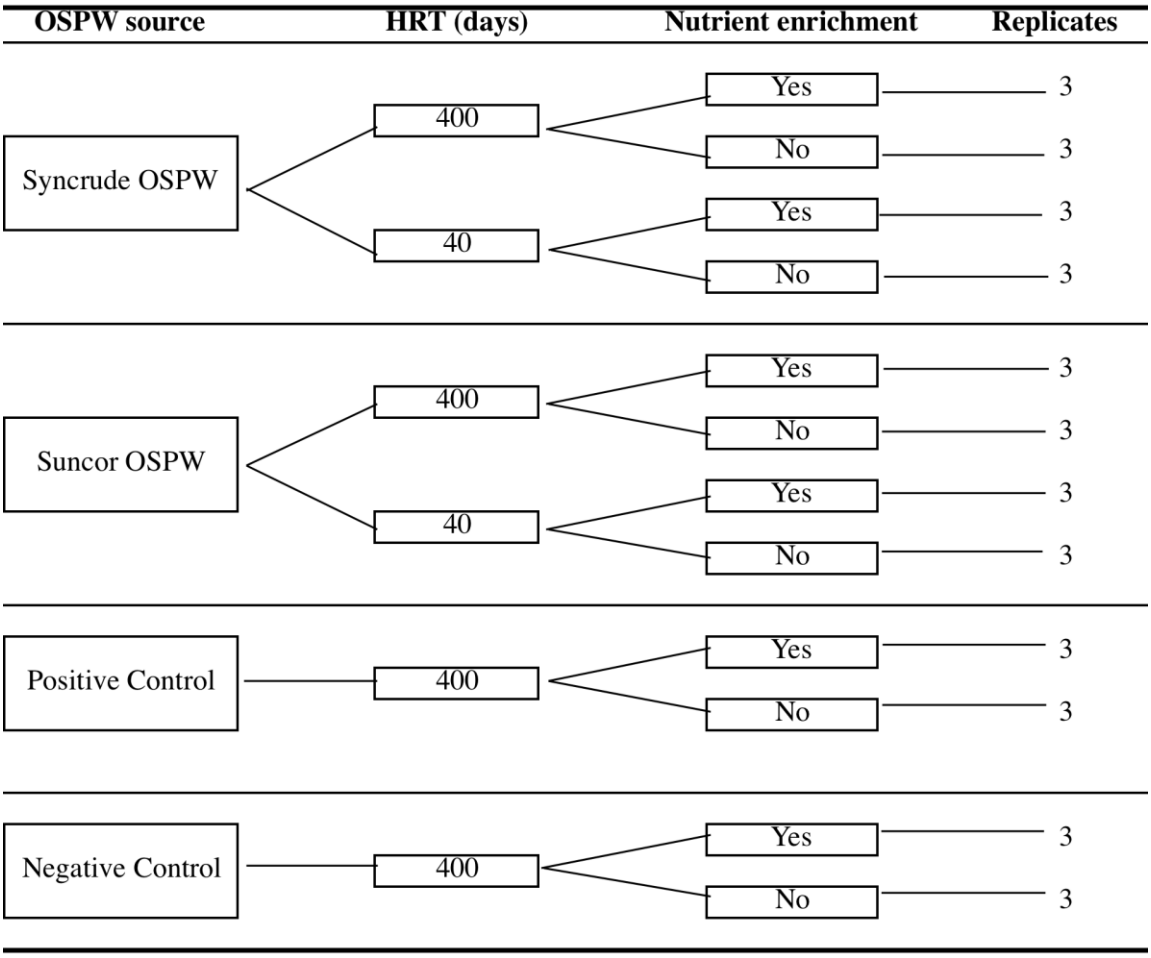


Figure 4.1. Experimental matrix for the 52-week simulated wetland laboratory microcosm study with oil sands process-affected waters (OSPW) maintained under two hydraulic retention times (HRT). Nutrient enrichment consisted of 5 mg/L $NaNO_3$ and 0.64 mg/L Na_2HPO_4.

Microcosms were evenly spaced on shelves in a controlled-environment chamber at the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada). Input waters for the 12 treatments were placed in 10-L (long HRT) and 20-L (short HRT) high density polyethylene plastic containers located above the experimental microcosms. A Masterflex® multi-channel peristaltic pump (Cole-Parmer Canada Inc., Montreal, QC, Canada) was used to continually pump the 12 treatment waters to their respective triplicate microcosms at two different rates to achieve the arbitrarily selected 40-day (short) and 400-day (long) HRTs. The 10-fold difference

in flow rates were achieved using two different sizes (3.1 and 0.8 mm I.D.) of Masterflex® high performance precision pump Tygon® tubing. Microcosms with a short HRT were recharged at a rate of 18 mL/h and microcosms with a long HRT were recharged at a rate of 1.8 mL/h, resulting in a residence time of about 40 and 400 days, respectively. Nitrogen and phosphorus, in the form of sodium salts (5 mg/L $NaNO_3$ and 0.64 mg/L Na_2HPO_4), were added to the input water containers of the six treatments with nutrient enrichment. All input water containers were covered with black plastic, and the tubes conducting the water to the microcosms were wrapped with duct tape to minimize light exposure and biological activity until the input water reached the test microcosms.

Environmental variables such as dissolved oxygen (DO) concentration, pH, temperature, and photoperiod were maintained at levels intended to promote microbial activity. The DO concentration in each microcosm was maintained between 2 and 8 mg/L by constant aeration. Microcosm pH was monitored regularly and on average was stable between 8.7 to 8.9, and 7.7 to 8.0, for those containing OSPW and control waters, respectively (Appendix A). The temperature of the test chamber was maintained at 25 ± 1°C. Additional fluorescent light bulbs, with a spectral range of 350 to 750 nm (Plant & Aquarium Brightstick® 33, Canadian Tire Corporation Ltd., Toronto, ON, Canada), were attached to the shelves directly above the microcosms. All chamber lighting was on a 16:8-hour light:dark photoperiod.

4.3.1.1 Simulated Wetland Microcosms

Thirty-six flow-through simulated wetland microcosms were fabricated at the Toxicology Centre using 20.8-L (40 x 20 x 25 cm) glass aquaria. Each glass aquarium was modified to include: a glass cover to reduce evaporation, a glass baffle to ensure better mixing of the water column, an aeration bubbler to maintain aerobic conditions, and both an inlet and outlet (1.3 cm diameter) to control the flow of OSPW (Figure 4.2). Each of the 36 aquaria contained approximately 18.9 L of water and 2.1 L of non-oil sands impacted wetland sediment (9:1 v/v).

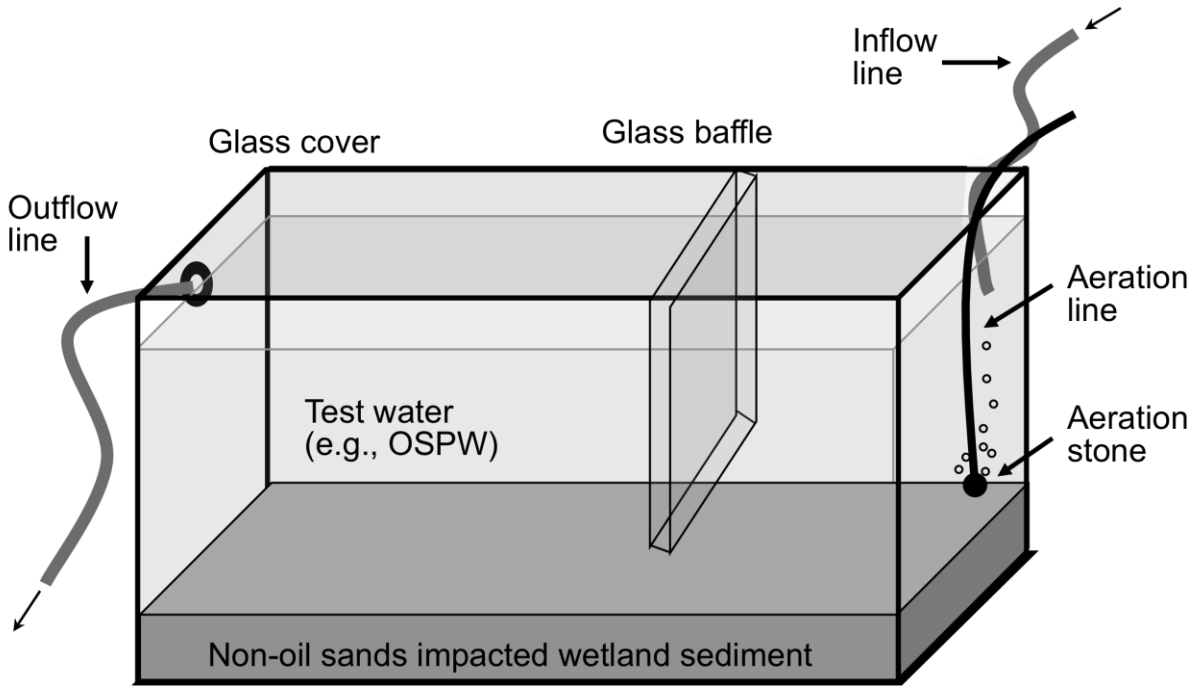


Figure 4.2. Schematic of a simulated wetland microcosm containing approximately 18.9 L of water and 2.1 L of non-oil sands impacted wetland sediment (9:1 v/v).

4.3.1.2 Test Waters and Sediment

Syncrude OSPW was collected periodically from October 12, 2006, to October 18, 2007, at a sand dyke drain located on Cell 25 of Syncrude's Mildred Lake Settling Basin (MLSB). The periodic collection of OSPW reflected any seasonal variability in OSPW composition and ensured a relatively fresh supply of test waters for the wetland microcosms. Mildred Lake Settling Basin is Syncrude's original settling basin located within Syncrude's Mildred Lake Site lease (Fort McMurray, AB, Canada). The seepage location, labelled DDB2506, is easily accessible and continually discharges OSPW that has slowly passed through the sand dyke under an anaerobic environment. This sand-filtered water is low in suspended solids and is comparable to "fresh" OSPW in terms of NAs, and represents the type of water that may some day be directed into wetland environments for reclamation. The mean chemical and toxicological properties of the waters used in this study are summarized in Table 4.1. In addition, the concentrations of the 16 United States Environmental Protection Agency (EPA) priority polycyclic aromatic hydrocarbons (PAHs) in Syncrude and Suncor OSPWs were all below their

respective detection limits which ranged from 0.01 to 0.05 µg/L (Appendix B). Suncor OSPW was collected from November 3, 2006, to October 18, 2007, at the discharge location of a wetland (Natural Wetland) located on the Suncor lease site, adjacent to Suncor Pond 2/3. The average residence time of Suncor OSPW in Natural Wetland is about 5 weeks, and this wetland receives a continuous recharge of sand dyke drainage waters from Pond 2/3 (OSPW) and non-OSPW from surface runoff at a ratio of about 3:1 (v/v) (W. Tedder, Suncor Energy Inc., Calgary, AB, Canada, personal communication).

Table 4.1. Properties (mean ± SD) of oil sands process-affected and control input waters used to charge and recharge the experimental microcosms.

Variable	Test water			
	Syncrude OSPW (n = 28)	Suncor OSPW (n = 28)	Positive[a] Control (n = 14)	Negative[b] Control (n = 14)
Source	Dyke drainage	Pond 2/3	CFMW	CFMW
pH	8.2 ± 0.4	8.6 ± 0.8	7.8 ± 0.3	8.2 ± 0.5
Conductivity (µS/cm)	2607 ± 225	1482 ± 238	589 ± 69	396 ± 34
Major Ions (mg/L)				
Na	687 ± 19	353 ± 55	77 ± 10	29 ± 1.5
Mg	14 ± 0.3	18 ± 6.4	18 ± 0.9	18 ± 1.2
Ca	17 ± 1.4	24 ± 9.2	31 ± 5.1	30 ± 3.0
Cl	227 ± 9.7	26 ± 3.6	66 ± 17	12 ± 2.3
SO_4	311 ± 13	274 ± 111	89 ± 7.0	91 ± 6.2
CO_3	22 ± 19	37 ± 69	<0.1	<0.1
HCO_3	1019 ± 53	638 ± 183	143 ± 21	121 ± 10
Naphthenic Acids (mg/L)	73 ± 9	58 ± 14	49 ± 19	<0.5
Microtox® Bioassay (% v/v)				
EC50	40 ± 19	77 ± 22	12 ± 4	>100
EC20	11 ± 6	22 ± 12	5 ± 2	>100

[a]Positive control water was prepared using carbon-filtered municipal water spiked with commercially availble Merichem naphthenic acids to a final concentration of approximately 75 mg/L.
[b]Carbon-filtered municipal water was used as the negative control.
OSPW = oil sands process-affected water.
CFMW = carbon-filtered municipal water
EC = effective concentration.

In addition to the test OSPW, positive and negative controls were included in the experimental design and maintained under similar conditions (Table 4.1). The positive control was prepared at the Toxicology Centre using Saskatoon carbon-filtered municipal water (i.e., non-OSPW) spiked with commercially available Refined Merichem Naphthenic acids (Merichem NAs; Merichem Chemicals & Refinery Services LLC, Houston, TX). Merichem NAs, which are typically less complex (i.e., lower average molecular weight) than OSPW NAs, have an acid number ranging from 200 to 260 mg KOH/g and a maximum unsaponifiable (i.e., non-carboxylic acid) matter content from 4 to 8% (w/w). Two grams of NaOH pellets were dissolved in 1 L of ultra-pure (Barnstead® NANOpure® Diamond$^{T.M.}$, Barnstead Intenational, Dubuque, IA) water to make a 0.05 M NaOH solution. Merichem NAs were then dissolved into this solution to make a 2 g/L NAs stock solution. Next, 375 mL of NAs stock solution was added to 9625 mL of carbon-filtered municipal water and mixed to achieve a final NAs concentration of approximately 75 mg/L. The pH of the positive control solution was adjusted to 8 using a 10% (v/v) hydrochloric acid (HCl) solution. Carbon-filtered Saskatoon municipal water was used as the negative control.

Sediment (top 10 cm of uppermost live vegetation and sediment) from a local, non-oil sands impacted wetland (Tower Road, west of Fort McMurray, AB) was collected on December 16, 2006. The sediment was primarily organic-rich silt and small organic debris, with trace amounts of sand. The total organic carbon (TOC) content of the wetland sediment was 27.6 ± 0.9% (mean ± SD; n =4). This material was shipped to the Toxicology Centre and stored at 4°C until it was homogenized and evenly distributed to all 36 microcosms as a substrate.

4.3.2 Sample Collection and Analysis

Input water from all containers supplying the microcosms (pre-treatment), and output water from the 36 microcosms (post-treatment, collected from inside the aquaria ~10 cm below the outlet), were collected at selected times during the experiment and stored in the dark at 4°C until analysis. Samples were shipped to the Syncrude Research Centre in Edmonton, AB, Canada, for NAs, trace metals, and major ions analyses following their standard methods (Syncrude Canada Ltd., 1995). Sample pH was measured with an Orion Model 835 pH meter (Beverly, MA), conductivity was measured with an Orion Model 170 Conductivity Meter (Orion Research Inc.,

Boston, MA), and hardness and alkalinity were measured using a HACH Digital Titrator Model 16900 (Hach Company, Loveland, CO). After filtration (0.45 µm Millex® syringe filters, Millipore Corporation, Bedford, MA), the concentrations of dissolved ions and minor elements were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Results from the general water chemistry, major ions, and trace elements analyses for the water collected from within the microcosms during the 52-week study period are summarized in Appendix A.

4.3.2.1 Naphthenic Acids Concentrations

Total NAs concentrations were measured using Fourier transform infrared (FTIR) spectroscopy, courtesy of Syncrude Canada Ltd. (Edmonton, AB, Canada). The procedure for quantifying NAs using FTIR spectroscopy is described in detail elsewhere (Jivraj et al., 1995; Holowenko et al., 2002). For each treatment water type, a single input water sample (pre-treatment), and output water samples (post-treatment) taken from each aquaria, were analyzed for total NAs concentration at time = 1, 13, 16, 25, 31, 36, 42, and 52 weeks.

Statistical differences between the mean total NAs concentrations in the input treatment waters and the output waters after 52 weeks for each treatment scenario, were determined using analysis of variance (ANOVA) followed by Holm-Sidak pair-wise comparisons. Comparisons were made within the treatment groups (Syncrude OSPW, Suncor OSPW, positive control) separately. Data were tested for Normality using the Shapiro-Wilk test (α =0.05) and for equal variance using the Levene Median test (α =0.05). All graphing and statistical analyses were conducted using SigmaPlot® software (Ver 11.0 Systat Software Inc.).

4.3.2.2 Aquatic Toxicity Bioassays

Rainbow trout (*Oncorhynchus mykiss*) 96-h static acute bioassays were conducted with both Syncrude and Suncor input waters, and with the waters present in the laboratory wetland microcosms after a 52-week exposure period. The tests were conducted by HydroQual Laboratories Ltd. (Calgary, AB, Canada) according to Environment Canada's test method (Environment Canada, 2000).

Microtox® bioassays were performed regularly on each treatment water type during the 52-week experiment. A single input water sample (pre-treatment) and output water samples (post-treatment) taken from each aquaria, were analyzed for toxicity at times = 1, 13, 16, 25, 31, 36, 42, and 52 weeks. The bioluminescent bacteria *Vibrio fischeri* was used to assess the toxicity of the samples. The 15 minute, 100% Basic Test was used to estimate the EC_{20} (effective concentration of the sample that results in 20% inhibition of bioluminescence in the test bacteria) for each sample using the Microtox® M500 Toxicity Analyzer (Azur Environmental, formerly Microbics Corporation, Carlsbad, CA) and related computer software (Microtox® Ver 7.11) (Azur Environmental, 1989).

Naphthenic acids are weak organic acids and thus the degree of proton dissociation and solubility depends upon their pK_a and the ambient pH of the surrounding medium. To control for differences in pH, the pH of all samples was adjusted to between 7 and 8 using 0.25 N HCl or 1% (v/v) NaOH prior to conducting a Microtox® bioassay. This adjustment reasonably standardized the NAs weak acid protonated and de-protonated equilibrium and allowed the bacteria *V. fischeri* to respond under optimum conditions as described in the Microtox® manual, A Toxicity Testing Handbook (Azur Environmental, 1989). Furthermore, two reference toxicants, phenol and zinc sulfate, were analyzed to verify the performance of the Microtox® system. All pH adjustments and testing of reference toxicants and samples were completed according to the manufacturer's protocol (Azur Environmental, 1989). The measured phenol and zinc sulfate EC_{50} values were always within their recommended ranges of 13 to 26 mg/L and 3 to 10 mg/L, respectively. Supplies for the Microtox® bioassay were purchased from Osprey Scientific (Edmonton, AB, Canada).

Linear regression analyses of Microtox® EC_{20} values versus time were performed on both input and output water samples for each treatment water type. The slopes and elevations (i.e., *Y* intercepts; vertical positions on graph) of the regression lines were tested for statistical significance according to methods for comparison of slopes based on analysis of variance (ANOVA) and the Student's *t*-test (Zar, 1999). The calculations were performed using Microsoft® Excel (2002). The regression analyses, graphing, and tests for normality (Shapiro-Wilk, α =0.05) were conducted using SigmaPlot® software (Ver 11.0 Systat Software Inc.).

4.4 Results and Discussion

4.4.1 Naphthenic Acids Concentrations

The treatment waters within the simulated wetland microcosms were maintained under aerobic conditions at a mean (±SD) temperature of 26.3 ± 1.0°C. A reduction in the total NAs concentrations were observed in Syncrude OSPW, Suncor OSPW, and the positive control, under all test conditions after 52 weeks. However, the overall extent of NAs degradation was limited to comparisons made between input and output (time = 0 vs. 52 weeks) OSPW NAs concentrations due to the observed fluctuation in NAs concentrations between time = 1 and 42 weeks. It is hypothesized that this variability was the result of NAs sorption onto the highly organic substrate (described in more detail below).

Syncrude OSPW showed a significant decrease in NAs concentrations between the initial input water and the output water at 52 weeks for both long and short HRTs and with and without nutrients (Figure 4.3). While nutrient supplementation appeared to stimulate the biological growth (based on visible observations of phytoplankton development) in the microcosms, there was no correlation with increased NAs removal. There was a greater reduction in NAs concentrations in the treatments with a long HRT (73 and 74% reduction, for Syncrude OSPW with and without nutrients, respectively; P <0.001) than in the treatments with a short HRT (29 and 40% reduction, for Syncrude OSPW with and without nutrients, respectively; P =0.016).

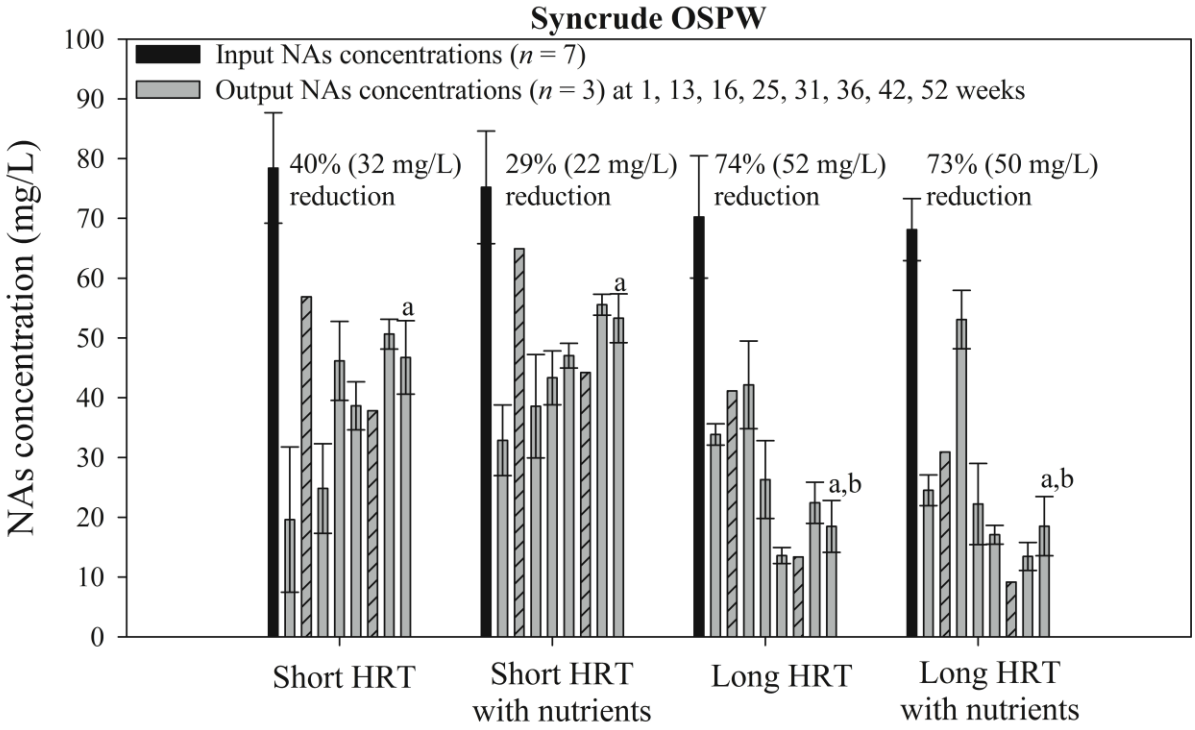


Figure 4.3. Total naphthenic acids (NAs) concentrations (mean ± SD) for the four Syncrude OSPW treatments (n =7 for input; n =3 for each output date). Hatched bars for the output water NAs concentrations at 13 and 36 weeks represent times when only one replicate was analyzed. The percent decrease and the absolute NAs concentration reduction (mg/L) between the average input water NAs concentration and the final NAs concentration at 52 weeks are presented above each treatment group. [a]Denotes a significant difference between the input treatment OSPW (black bar) and the same OSPW at 52 weeks (final grey bar). [b]Denotes a significant difference between the long and short hydraulic retention time (HRT) treatments at 52 weeks (final grey bars).

In microcosms receiving Suncor OSPW there was also a significant decrease in NAs concentrations between the initial input water and the output water at 52 weeks for the treatments under a long HRT and with and without nutrients (Figure 4.4). Similar to Syncrude OSPW, nutrient supplementation in Suncor OSPW was not correlated with increased NAs removal. The degree of NAs reduction in the output water after 52 weeks relative to the input water was dependent on the HRT (P =0.008). Overall, there was a greater reduction in NAs concentrations in treatments with a long HRT (64 and 74% reduction for Suncor OSPW with and without

nutrients, respectively; P =0.001) than in treatments with a short HRT (25 and 30% reduction for Suncor OSPW with and without nutrients, respectively; P =0.851).

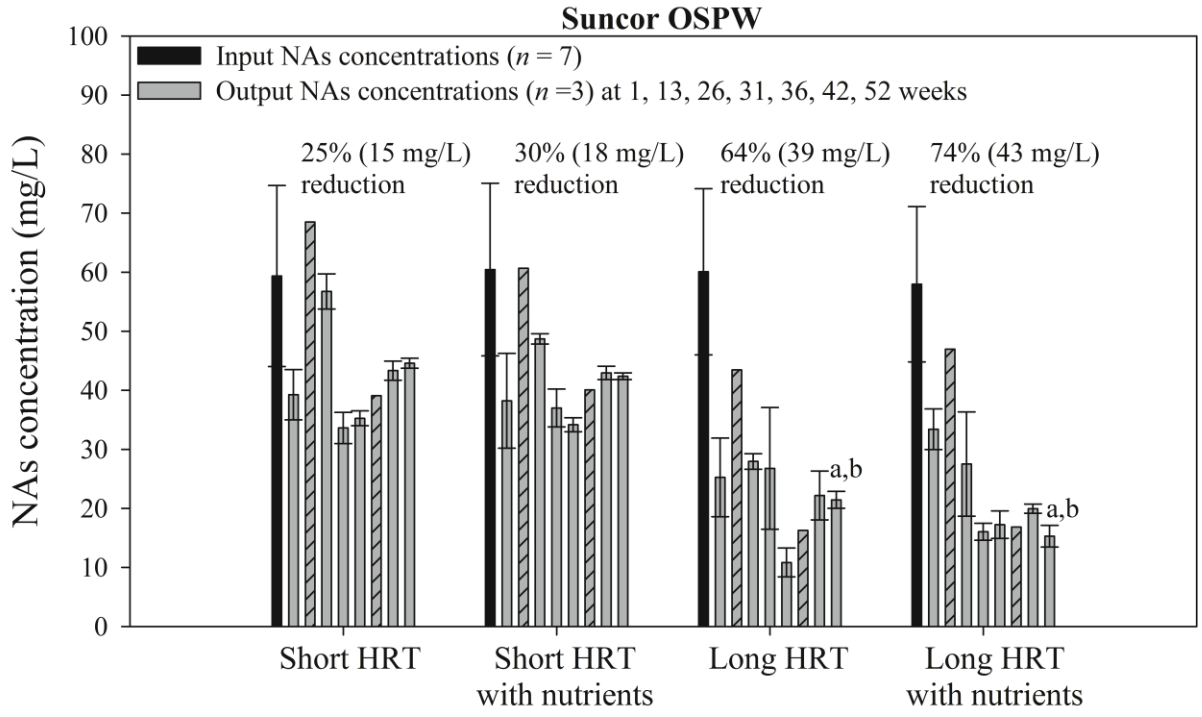


Figure 4.4. Total naphthenic acids (NAs) concentrations (mean ± SD) for the four Suncor OSPW treatments (n =7 for input; n =3 for each output date). Hatched bars for the output water NAs concentrations at 13 and 36 weeks represent times when only one replicate was analyzed. The percent decrease and the absolute NAs concentration reduction (mg/L) between the average input water NAs concentration and the final NAs concentration at 52 weeks are presented above each treatment group. [a]Denotes a significant difference between the input treatment OSPW (black bar) and the same OSPW at 52 weeks (final grey bar). [b]Denotes a significant difference between the long and short hydraulic retention time (HRT) treatments at 52 weeks (final grey bars).

The interaction between time (i.e., exposure within the microcosm) and HRT (i.e., throughput or flow of recharged test waters) affected the degree of NAs degradation in OSPW. In this study, the faster recharged wetlands (i.e., short HRT) reduced the contact time between NAs and the NAs-degrading microorganisms within the microcosms. However, given longer exposure times with the long HRT treatments (lower OSPW recharge rates), greater NAs reductions were

observed. After 52 weeks, total NAs concentrations in both the Syncrude and Suncor OSPWs, maintained under the long HRT with and without nutrients, were reduced to an average final concentration of 18 ± 1 mg/L (mean ± SE, n =4).

It is possible that total NAs concentrations would have continued to decrease if the study had been allowed to continue for longer than 52 weeks. However, the general trend over the final few sampling time points suggested that the greatest reduction in the NAs concentrations occurred after 31 to 36 weeks, with a lack of further reduction observed over the ensuing 21 weeks, suggesting a resistance to further degradation. Thus, even with a long HRT (e,g., greater than one year under controlled environmental conditions) a fraction of the NAs mixture is expected to resist degradation, or degrade at a much slower rate, and thus would potentially persist in the water column of aquatic reclamation environments. This is consistent with field observations made at Syncrude Canada Ltd. where OSPW transferred to experimental test ponds, isolated from fresh OSPW input, and naturally aged for up to 11 years, contained NAs concentrations of approximately 20 mg/L (Leung et al., 2001; Holowenko et al., 2002; Han et al., 2009).

Differences in the structural characteristics of the residual NAs compounds relative to the readily biodegradable NAs fractions may contribute to the observed resistance to microbial degradation. The analytical method used in this study to quantify the NAs was the FTIR method (Jivraj et al., 1995). The FTIR method of analysis is based on the measurement of the IR spectra related to the carboxyl group and thus is limited in its ability to determine structural or compositional characteristics of complex NAs mixtures. More advanced analytical techniques such as the High Performance Liquid Chromatography/Quadrupole Time of Flight-Mass Spectrometry (HPLC/QTOF-MS) analysis can enhance resolution by characterizing NAs based on carbon number and Z series (i.e., number of rings in a NA congener) (Bataineh et al., 2006; Han et al., 2008). This technique has been utilized in parallel investigations.

It is speculated that the observed reductions in NAs concentrations were strongly influenced by the aerobic conditions and natural biology of the sediment and water. The microorganisms indigenous to both OSPW and the wetland sediment were presumed to be primarily responsible

for the observed biodegradation of NAs in this study. However, the influence of sorptive processes on the overall reduction in dissolved NAs could not be discounted. While NAs in OSPW have low sorption coefficients (K_d) (e.g., <0.5 on sand and between 1 and 3 on different clay substrates; Schramm et al., 2000), Janfada et al. (2006) showed that soils having a higher organic carbon content have increased NAs adsorption. The K_d values for OSPW NAs were found to be as high as 17.8 mL/g in synthetic, high salinity (total ion concentration greater than 2400 mg/L) groundwater at a pH of approximately 8. The high K_d value was attributed to a decrease in NAs solubility due to the increased availability of binding sites on the soil (i.e., the organic fraction of the soil), and the potential "salting-out" of NAs in the presence of elevated levels of Na^+, Ca^{2+}, Mg^{2+}, and SO_4^{2-} ions (Janfada et al., 2006). The simulated wetland microcosms used in this research contained high salinity OSPW (e.g., total dissolved solids of 1000 to 2000 mg/L; Table 4.1) and a highly organic sediment substrate (27.6% TOC). These two conditions could have promoted some NAs adsorption, especially during the initial stages of the study when OSPW was introduced into the aquaria, even at the ambient pH values of 8.7 to 8.9.

Results from the FTIR analysis (Figures 4.3 and 4.4) supports the hypothesis that OSPW NAs in the microcosms might have undergone initial rapid sorption. Reductions in NAs concentrations between the input water and the measurement taken from the microcosms at 1 week occurred for all OSPW treatments regardless of HRT. Although steps were taken to minimize the disturbance of sediments, some re-suspension was observed in all aquaria, which required approximately 5 days to completely re-settle, providing sufficient time for NAs sorption to occur. The NAs concentrations, especially in the treatment with Syncrude OSPW and a short HRT, appeared to increase slightly over time after this initial reduction. It is hypothesized that sorption on the highly organic sediment may have occurred initially, but eventually binding sites were largely saturated and this effect mitigated (thus more NAs from the input water subsequently stayed in the water column). There appeared to have been only a minor influence of biodegradation in the treatments with short HRT, or it was offset by the continuous renewal of NAs as substrate binding sites were saturated. The OSPW residence time in these microcosms was apparently inadequate for significant biodegradation to be observed.

In the treatments with long HRT, a sorption effect was likely also present, but overshadowed by an increased rate of biodegradation and slower NAs renewal. Here the initial sorption appeared to be the same as for the short HRT, but NAs concentrations now generally decreased, as there was also adequate residence time for biodegradation to occur. Determining the influence of sorption of NAs was not a primary objective of this study, but was subsequently investigated (see Chapter 6). Regardless, the observed reduction of NAs can be considered as an overall "wetland" type dissipation mechanism, which would include various biological, chemical, and physical processes.

In contrast to the microcosms receiving OSPW, the loss of NAs from the positive controls that contained the commercial NAs mixture, with and without nutrient addition, was essentially complete after 52 weeks. The NAs concentrations were significantly reduced by 16 weeks (P <0.05) (Figure 4.5). Regardless of treatment conditions, there was greater than 98% reduction in the commercial NAs after 52 weeks, and total NAs concentrations in the discharge waters were below 1 mg/L.

There was a significant interaction between the effect of time (i.e., the difference in NAs concentration between the initial input water and the output water at 16 weeks) and the addition of nutrients in the positive control groups. This interaction was a result of the significant difference between the mean NAs concentration in the input water of the positive control containing nutrients and that without nutrients (P <0.001). On average, the input water NAs concentration was 44% (28 mg/L) lower in the treatment supplemented with nutrients than in the treatment without and was speculated to be a result of the difficulties associated with the dissolution of NAs.

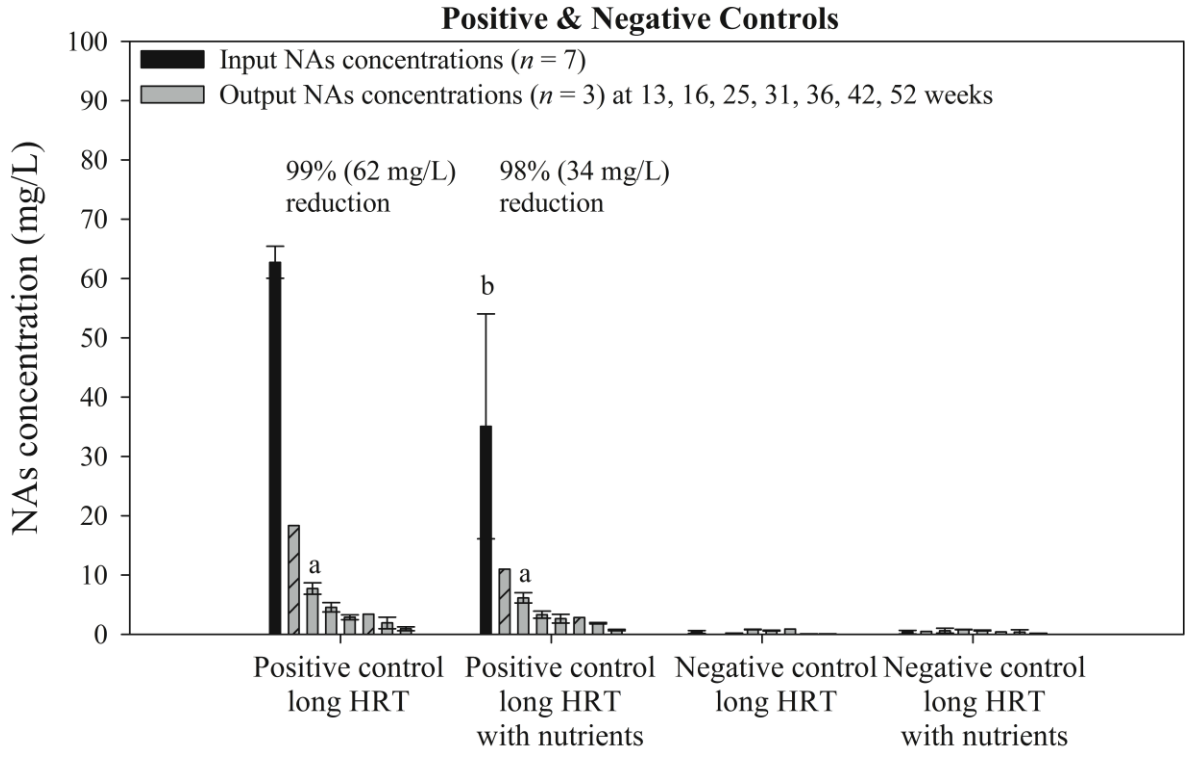


Figure 4.5. Total naphthenic acids (NAs) concentrations (mean ± SD) for the two positive and two negative control treatments (n =7 for input; n =3 for each output date). Hatched bars for the output water NAs concentrations at 13 and 36 weeks represent times when only one replicate was analyzed. The percent decrease and the absolute NAs concentration reduction (mg/L) between the average input water NAs concentration and the final NAs concentration at 52 weeks are presented above each treatment group. [a]Denotes a significant difference between the NAs concentration in each input positive control treatment (black bar) and the same positive control at 16 weeks (grey bar). [b]Denotes a significant difference between the input NAs concentrations in the positive control treatment water with and without nutrients (black bars). Negative controls were not assessed statistically.

The present study confirmed previous findings that there was no, or only a negligible, persistent fraction associated with commercial NAs. Herman et al. (1994b) and Scott et al. (2005), using different laboratory microcosms inoculated with mixed bacterial cultures, reported greater mineralization of commercial NAs compared to NAs extracted from tailings pond water. These two studies also demonstrated that the NAs mixture from tailings pond waters contained persistent components that were not observed in commercial NAs mixtures. The possibility of interfering constituents in OSPW capable of reducing NAs biodegradation rates was ruled out by

Scott et al. (2005). The readily biodegradable nature of the commercial NAs relative to OSPW NAs in the present and aforementioned studies is likely due to the source and compositional differences of the mixtures. Regardless, the positive control treatment successfully demonstrated the effectiveness of the microcosms in reducing the concentration of commercial NAs with characteristics similar to at least some OSPW NAs.

The NAs found in OSPW are derived from bitumen. The bitumen associated with the oil sands, prior to extraction by the operators, will already have undergone extensive biodegradation in geologic environments exposed to ground and surface waters (Tissot and Welte, 1984). The relative recalcitrant nature of OSPW NAs compared to that observed with commercial NAs may thus be related to the long-term biodegradation processes that OSPW NAs have already undergone before being released into the OSPW during oil sands extraction. Conversely, commercial NAs are typically derived as petroleum distillate fractions from conventional crude oil (Brient et al., 2000) that can be considered young and un-weathered relative to the highly weathered bitumen in the AOS. Studies have shown that commercial NAs mixtures biodegrade faster (Herman et al., 1994b; Clemente et al., 2004; Scott et al., 2005) and consist of more lower molecular weight compounds than OSPW NAs (Headley and McMartin, 2004). The former studies support the hypothesis that NAs mixtures from tailings pond waters contain persistent components that are not found in commercial NAs. Thus, the slower degradation kinetics of OSPW NAs, not unlike those observed in this microcosm study, are likely due to the presence and persistence of the larger molecular weight compounds.

Herman et al. (1993) noted that microbial activity in oil sands tailings pore water was both nitrogen- and phosphorus-limited. Subsequently, Lai et al. (1996) demonstrated that phosphate addition increased the rate of degradation of model NAs dissolved in tailings pond water. In contrast, the addition of nutrients in this study did not significantly enhance NAs degradation in either OSPW, or in the positive control. Contrary to the test materials of Lai et al. (1996), the water and sediments in the microcosms used in this study may already have contained concentrations of nutrients greater than what was required to enhance NAs degradation. Syncrude and Suncor OSPW were collected in the field from sources representative of what might be expected as recharge waters to aquatic reclamation environments. Leung et al. (2001)

have shown that OSPW from Syncrude can contain total phosphorous and nitrogen concentrations greater than 0.180 and 10 mg/L, respectively. In addition, the sediment used as microcosm substrate was collected from a productive local wetland and contained a high volume of organic matter, mostly in the form of vegetation. Although actual nutrient concentrations within the sediment were not determined, this substrate could also have acted as a source of nutrients (Horne and Goldman, 1994) for the microorganisms in the microcosms. Given the potentially high background concentrations of nutrients in the OSPWs and microcosm sediments, nutrients were likely not limited. This suggests that further nutrient enrichment may not be needed in operational reclamation wetlands if such are constructed with a nutrient-containing substrate.

4.4.2 Aquatic Toxicity Bioassays

The initial acute toxicity to rainbow trout of input Syncrude OSPW (LC_{50} =67% v/v, 95% C.I. =57 to 79%) was completely removed after being added to the simulated wetland microcosms for 52 weeks under both long and short HRTs (LC_{50} >100% v/v). Fish survival in 100% (v/v) input Syncrude OSPW (pre-treatment) and 100% (v/v) output microcosm water (post-treatment) increased from 0 to 100%. Conversely, there was no acute toxicity associated with both input and output Suncor OSPW (LC_{50}s >100% v/v). Therefore, since total NAs concentrations between Syncrude and Suncor OSPW were similar, other factors such as NAs mixture composition must have influenced the aquatic toxicity of the two OSPWs.

The biodegradation of NAs has been associated with a reduction in acute toxicity in a number of previous studies (Herman et al., 1994b; Lai et al., 1996; Schramm et al., 2000; Holowenko et al., 2002; Clemente et al., 2004). Also, a bench-scale aerobic bacterial degradation study using Suncor tailings pond water (Nix and Martin, 1992) demonstrated that the acute toxicity, as measured by the survival of rainbow trout fingerlings, was completely removed within 6 to 12 weeks. Temperatures between 10 and 20°C and small amounts of dissolved oxygen (1 to 3 mg/L) were effective at stimulating the detoxification process. Similarly, the reduction in the acute toxicity of Syncrude OSPW to rainbow trout in this research may have been enhanced by the continuous aeration (dissolved oxygen = 4.9 ± 0.8 mg/L) and increased temperature (26.3 ± 1.0°C) present in the microcosms, which in turn, may have increased the biodegradation of NAs.

Reduction in rainbow trout toxicity observed under both long and short HRTs, even with continuous OSPW recharge, suggests that wetlands and wet landscape reclamation environments may be quite effective in the removal of acute OSPW toxicity to fish.

It is interesting to note that the input Suncor OSPW, with an average NAs concentration of 60 mg/L, did not display any initial acute toxicity to rainbow trout. This may be the result of the greater natural degradation of Suncor OSPW. The Suncor OSPW was drawn from a wetlands exposed to natural seasonal cycles. While it consisted of mainly OSPW originating as seepage waters from the dyke system of an adjacent settling basin, it also reflects some natural ageing in the wetlands (~5 weeks) and some dilution from precipitation runoff (25 to 40% v/v). This could be sufficient to reduce the acute toxicity to rainbow trout.

The partial, natural degradation of this Suncor OSPW may also have contributed to structural and compositional changes in the NAs mixture that resulted in the partial detoxification of OSPW while still retaining a significant total NAs concentration. For instance, Bataineh et al. (2006) showed that the mixture of NAs in Syncrude OSPW contain more highly branched isomers than commercial NAs, and that tailings water microorganisms preferentially deplete the least alkyl-substituted fraction, leaving behind more highly branched isomers. Moreover, a recent microbial NAs degradation study by Han et al. (2008) showed that the half-lives of OSPW NAs (44 to 240 days) were much greater than the half-lives of commercial NAs (1 to 8 days) and that this persistence was related to the higher degree of alkyl branching of OSPW NAs. It is thus possible that the NAs in Suncor OSPW, due to previous microbial exposure in the wetland, have a higher proportion of these highly branched NA isomers than the NAs in Syncrude OSPW. Or similarly, that the NAs in the two OSPWs are branched to a greater extent than the commercial NAs. Therefore, complete removal of NAs may not be required to reduce the acute toxicity of OSPW to fish, and the transformation of the more acutely toxic NAs homologues to congeners with higher degrees of branching (as an intermediate step to complete mineralization) may contribute to the detoxification process for acute toxicity, independent of the final total NAs concentration.

For the input OSPWs used in this study, the Microtox® results showed minimal acute toxicity (based on EC_{50}s). For this reason, assessment focused on changes in the EC_{20}, which can be considered more of a sublethal or chronic toxicity response (MacKinnon and Boerger, 1986). However, the reduction in total NAs concentrations, in both Syncrude and Suncor OSPW directed through the simulated wetland microcosms, did not result in a significant decrease in the Microtox® EC_{20} even in the treatments with long HRTs.

Regression analysis of the Microtox® EC_{20}s and time for Syncrude OSPW (Figure 4.6) indicated that toxicity of both input and output waters did not change significantly (P >0.05) over the 52-week test period (i.e., no significant differences in slopes from zero), and that the toxicity was the same in both input and output OSPW (i.e., no significant differences in the Y-intercepts of the regression lines, P >0.05) after accounting for the effect of time. Treatments with increased retention time and nutrient enrichment did not appear to enhance the reduction in the observed chronic toxicity in the microcosm discharge waters. Similarly, both the input and output toxicity of Suncor OSPW (Figure 4.7) did not change significantly (P >0.05) during the 52-week experiment, and the toxicity was generally the same in both input and output OSPW (P >0.05). However, there was one instance where the EC_{20}s of the output Suncor OSPW were statistically higher than the EC_{20}s of the input water, suggesting that some detoxification may have occurred. This occurred in the treatment with a short HRT and no nutrient addition, and not in the long HRT treatment as may have been expected. In theory, the treatments with the longest residence times should have allowed for the greatest opportunity for a reduction in toxicity. Given that this difference was not observed in either treatment with a long HRT, the significance of this observation was difficult to establish. No chronic toxicity (EC_{20} >100% v/v) of the output water from the positive controls, with and without nutrients, was observed after 16 weeks in the simulated wetland microcosms (Figure 4.8).

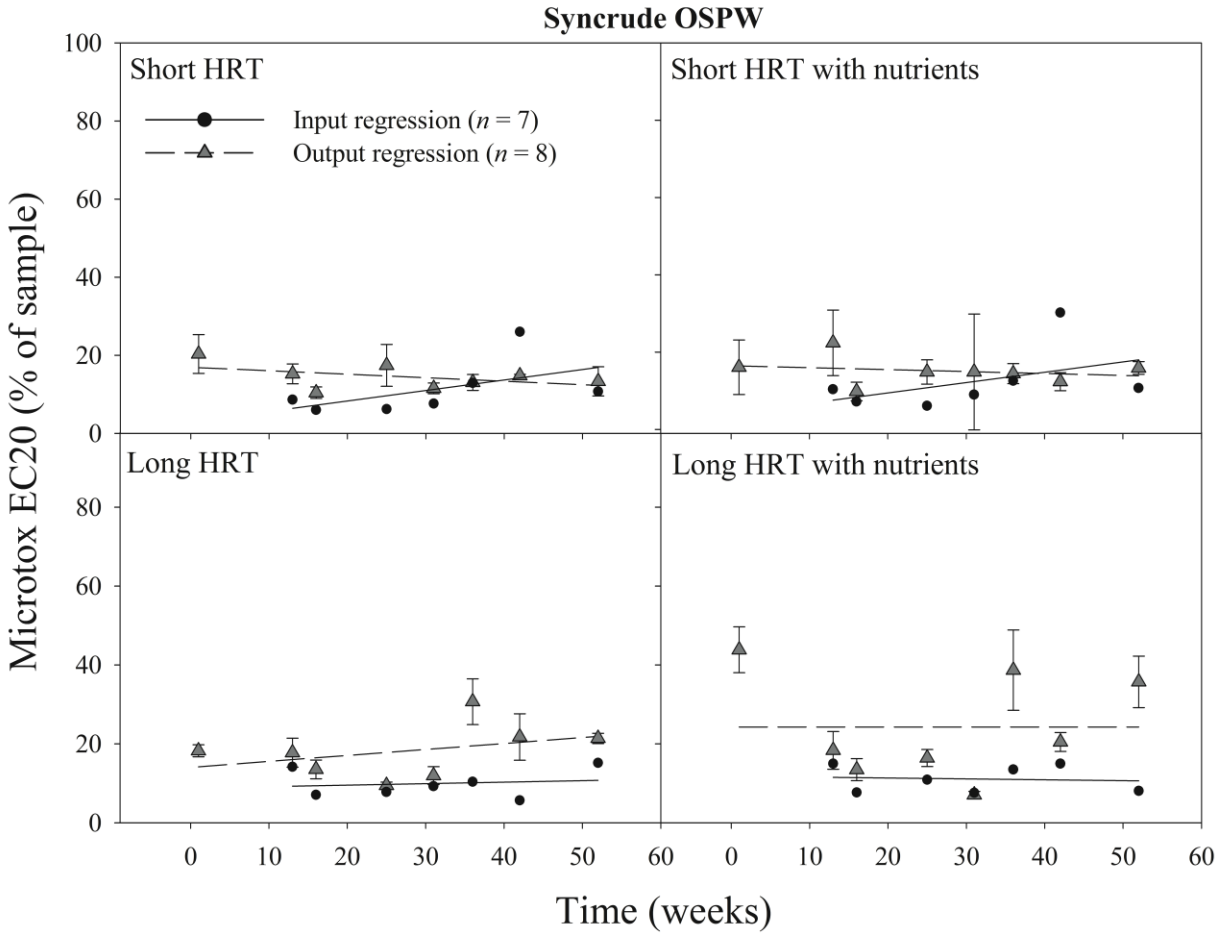


Figure 4.6. Linear regressions of input and output water Microtox® EC_{20} values (mean ± SD; *n* =3 for output EC_{20} values; *n* =1 for input EC_{20} values) vs. time for all treatments with Syncrude OSPW.

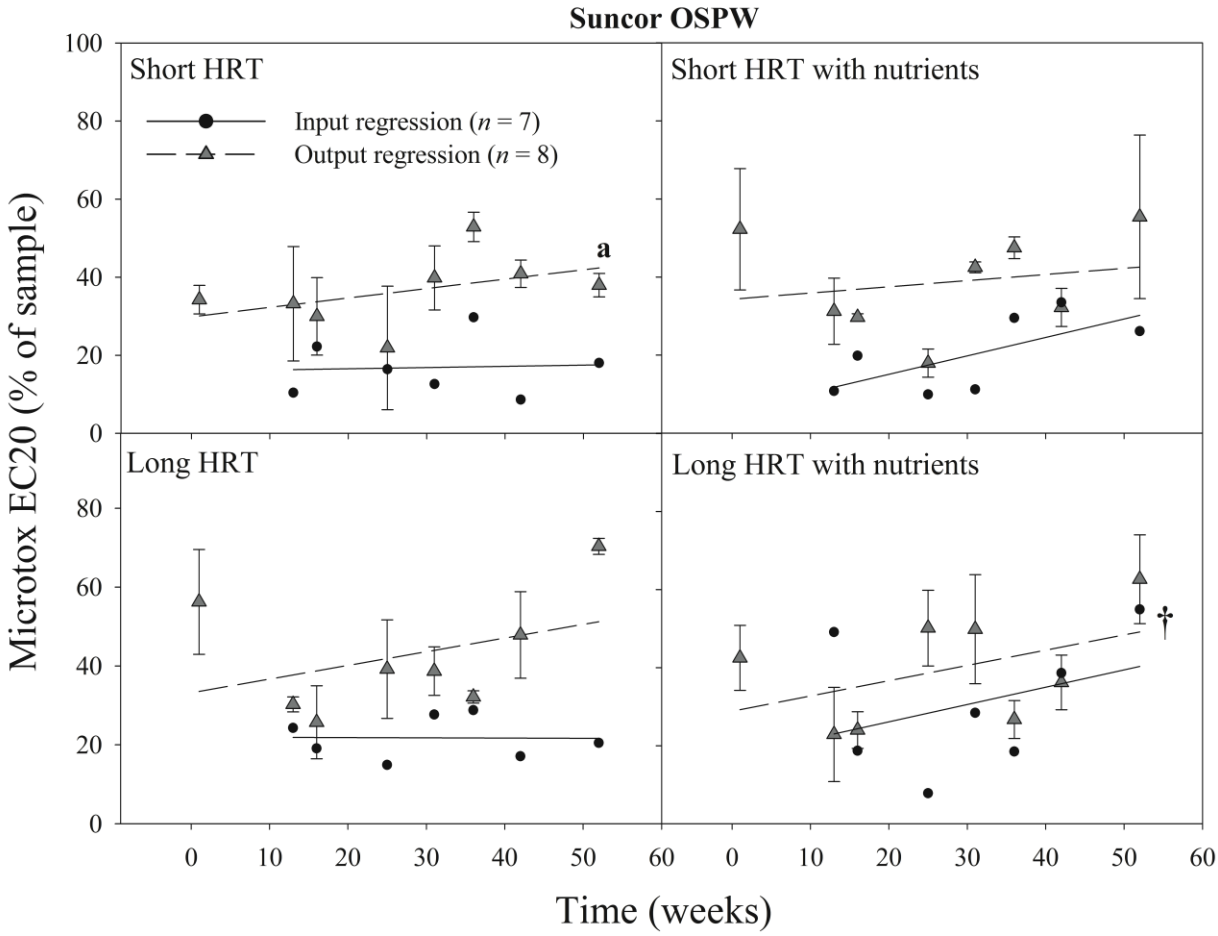


Figure 4.7. Linear regressions of input and output water Microtox® EC_{20} values (mean ± SD; n =3 for output EC_{20} values; n =1 for input EC_{20} values) vs. time for all treatments with Suncor OSPW. [a]Denotes a significant difference between the toxicity of the input and output waters in the short hydraulic retention time (HRT) (without nutrient addition) treatment scenario (P <0.05). [†]Data for the output water Microtox® EC_{20} values in the treatment with a long HRT and nutrient addition failed the test for normality (P =0.039).

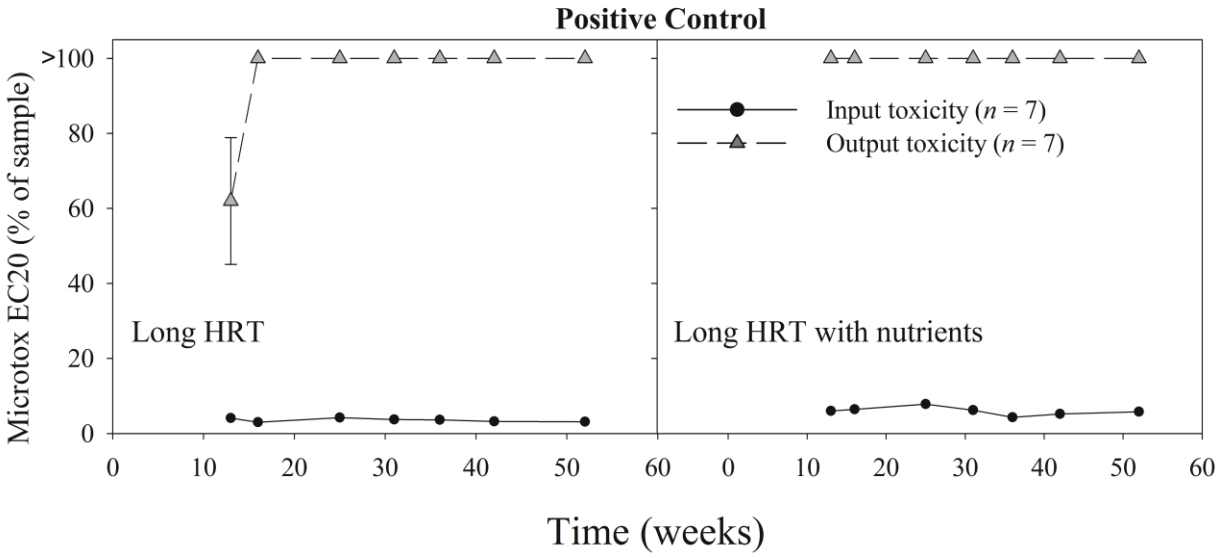


Figure 4.8. Input and output water Microtox® EC_{20} values (mean ± SD; n =3 for output EC_{20} values; n =1 for input EC_{20} values) for the positive control treatments during the 52-week test period.

The observed Microtox® toxicity (based on EC_{20}s) was unchanged in both OSPWs regardless of the HRT, the addition of nutrients, or the significant reduction observed in total NAs concentrations. This result is consistent with those obtained during the long-term ageing of OSPW in Syncrude's Experimental Test Pond facility (Han et al., 2009). Due to the lack of an observed concentration-response relationship, especially in the treatments with a long HRT, the persistent or chronic toxicity of OSPWs appears to be related to a persistent fraction of NAs that had not, or had minimally, dissipated or degraded after 52 weeks. Lai et al. (1996) presented similar results where the biodegradation of representative NAs by tailings-water microorganisms resulted in a decrease in acute toxicity of tailings pond water to fathead minnows (*Pimephales promelas*), but not in toxicity to the bioluminescent bacteria used in the Microtox® bioassay (reported as an EC_{20}). This is also consistent with other studies conducted in the field and in the laboratory that showed some removal of acute toxicity after biodegradation and the persistence of residual chronic toxicity (Nix and Martin, 1992; Herman et al., 1994b; Holowenko et al., 2002). It is possible that some compounds present in OSPW other than NAs may have contributed to the observed toxicological response. However, based on the results for NAs

concentrations and other measured constituents, including ammonia, it appears that acute OSPW toxicity is associated with the readily biodegradable fraction of NAs, or the acid-extractable fraction, and that chronic OSPW toxicity is related to the more persistent organic compounds within a given NAs mixture.

The mechanism of NAs toxicity is not well understood. As a result of their surfactant properties, narcosis has been suggested as the primary mode of action for NAs acute toxicity (Frank et al., 2008). Narcosis in aquatic organisms has been defined as a nonspecific reversible alteration of the functioning of membranes caused by the accumulation of narcotic chemical in the hydrophobic phases or lipid bilayers of membranes (Wezel and Opperhuizen, 1995). Narcosis by polar chemicals can result from their accumulation at the membrane/water interface due to the influence of a strong hydrogen-bonding group, as opposed to a slightly dissimilar mode of action of nonpolar narcotics that can accumulate within the hydrophobic core of the membrane via hydrophobic interactions (Wezel and Opperhuizen, 1995). Naphthenic acids are amphiphilic molecules containing a hydrophilic carboxylic acid moiety and a hydrophobic alkyl group (Clemente and Fedorak, 2005) and could potentially interact with membranes and contribute to toxicity by either hydrogen-, or hydrophobic-bonding depending on the charge of the molecules. Although both ionized and non-ionized forms of organic acids are absorbable (Zhao et al., 1996), generally the ionized substance enters a cell at a much slower rate, and therefore it is assumed that only the non-ionized forms significantly contribute to the toxicity, especially in the absence of active transport (Seward and Schultz, 1999).

Previous studies have shown that the acute aquatic toxicity of OSPW NAs mixtures is mainly driven by the smaller lower molecular weight compounds (Holowenko et al., 2002; Frank et al., 2008). In addition, Bataineh et al. (2006) assessed the chromatographic behavior of NAs injected onto a capillary reversed-phase HPLC column and found that NAs isomer classes with decreasing cyclization had longer retention times. Thus, it is plausible that some of the smaller molecular weight NAs with fewer rings are more hydrophobic and thus have the ability to more effectively interact with cellular membranes via hydrophobic interaction, while at the same time have a decreased influence from the charge on the carboxylic moiety. In contrast, heavier molecular weight NAs with increased cyclization, have shorter retention times and appear to be

less hydrophobic (Bataineh et al., 2006). Recently, Frank et al. (2009) showed that the carboxylic acid content increased in higher molecular weight fractions of OSPW NAs mixtures and that this increased carboxylic acid content could impair uptake into cellular membranes (Frank et al., 2010). Similarly, QSAR analysis of representative aliphatic carboxylic acids (Seward and Schultz, 1999) and NA-like surrogates (Frank et al., 2010), have shown that di-carboxylic acids are less toxic than mono-carboxylic acids. This would again explain why smaller molecular weight NAs have a higher acute toxicity potential relative to the more complex heavier molecular weight NAs.

Although differences in the structural complexity of the different NAs are not described herein, such differences provide a plausible explanation for the observations made in this study with respect to differences in the acute toxicities of the three treatment water types. The commercial NAs, believed to be more hydrophobic and have the least complex structures, were the most acutely toxic and the most readily detoxified after addition to the wetland microcosms. Similarly, commercial NAs have been shown to be more acutely toxic to yellow perch (*Perca flavescens*) than NAs extracted from OSPW (Nero et al., 2006a). The NAs present in Syncrude and Suncor OSPW were suspected of containing both a readily degradable, more hydrophobic fraction associated with the easily detoxified acute toxicity similar to that of the commercial NAs, and a persistent fraction containing more complex structures with increased carboxylic acid content accounting for the reduced degradation and persistent chronic toxicity.

4.5 Conclusions

The simulated wetlands used in the current study provided a simple analogue of constructed wetland performance as a treatment option for OSPW in lease closure landscapes. The passage of OSPW through a wetland environment that provides sufficient time and maintains aerobic conditions should be capable of reducing total NAs concentrations in both Syncrude and Suncor OSPW. Removal of some NAs in the microcosms was related to a decrease in acute toxicity of OSPW to rainbow trout. While dissipation of some NAs was evident in all treatments, it was clear that relative to the shorter HRT (40 days), the longer HRT (400 days) resulted in a greater reduction in total NAs in both OSPWs. While residence time under aerobic conditions seems to be critical to bioremediation success in wetland habitats, the addition of nutrients may not result

in a significant enhancement of treatment effectiveness. Furthermore, neither nutrient addition nor extended residence times (long HRT) resulted in the complete removal of the persistent fraction of NAs, or the persistent toxicity of OSPW (Microtox® EC_{20}s). According to this research, OSPW reclamation at the AOS using wet landscapes would require hydraulic residence times that were sufficiently long to effectively reduce the concentration of the persistent fractions of NAs and the associated chronic toxicity. Whether that is possible under "normal" conditions (without supplemental treatment) is presently unknown.

5.0 SELECTIVE BIODEGRADATION OF NAPHTHENIC ACIDS AND A PROBABLE LINK BETWEEN MIXTURE PROFILES AND AQAUTIC TOXICITY

This chapter will be submitted with minor revision for publication in the journal Environmental Toxicology and Chemistry. The full citation will be: Toor, N.S., Han, X., Franz, E.D., MacKinnon, M.D., Martin, J.W., Liber, K. Selective biodegradation of naphthenic acids and a probable link between mixture profiles and aquatic toxicity. Environmental Toxicology and Chemistry.

5.1 Abstract

The toxicity of oil sands process affected waters (OSPW) from the Athabasca Oil Sands (AOS) in northern Alberta, Canada, is related to a relatively persistent group of dissolved organic acids known as naphthenic acids (NAs). Naphthenic acids are a complex mixture of saturated alkyl-substituted acyclic and cyclic aliphatic carboxylic acids, with a general formula $C_nH_{2n+z}O_2$, where *n* indicates the carbon number and *Z* specifies the number of rings in the molecule. This research, evaluated the potential for the selective biodegradation of NAs and the associated reduction in aquatic toxicity of OSPW using flow-through laboratory wetland microcosms over a 52-week test period. High performance liquid chromatography/quadrupole time of flight-mass spectrometry (HPLC/QTOF-MS) analysis was used to track the changes in NAs mixture profiles or 'fingerprints' in each treatment over time. Based on first order degradation kinetics, more rapid degradation was observed for NAs that had lower carbon numbers and fewer degrees of cyclization (NAs congeners with carbon numbers 11 to 16 and *Z* series -2 to -4; half-lives between 19 to 28 weeks). Within the NAs mixture fingerprint, the two most persistent groups of homologues were also identified (NAs with carbon numbers 17 to 20 and *Z* series -6 to -12; half-lives between 37 to 52 weeks). The persistence of this group of NAs may aid in explaining the residual chronic toxicological response as measured by the Microtox® bioassay (EC_{20}), after the degradation of the more labile fractions of NAs mixtures in OSPW.

5.2 Introduction

The Athabasca Oil Sands (AOS) in northeastern Alberta, Canada, contain sizeable reserves of bitumen recoverable using surface mining and *in-situ* operations (Frank et al., 2009). Production of crude oil, using integrated surface mining methods, involves open-pit surface mining and transport of the oil sand ore to an extraction plant. Here, the bitumen is separated from the sand using the Clark caustic hot water extraction process (Schramm et al., 2000). The extracted bitumen is then upgraded, a process that converts it from a heavy, viscous oil to a sweet, light synthetic crude oil ready for further refining using more conventional techniques (Singh, 1985). Commercial development has been underway in this region since the late 1960s and total bitumen production has grown from 1.49 million barrels per day in 2009 to 1.61 million barrels per day in 2010 (Alberta Energy and Utilities Board, 2010-2011).

The extraction and upgrading processes of current surface mining operations use approximately 2 to 2.5 m^3 of fresh water to produce 1 m^3 of synthetic crude oil (W. Zubot, Syncrude Canada Ltd., Edmonton, AB, personal communication). Contact of water with the ore and the resulting leaching during extraction, as well as the addition of process aids such as sodium hydroxide, leads to a loading of dissolved inorganic and organic constituents into process waters. Aggressive recycling of site waters further affects the water inventory within the tailings deposits and retention ponds. The site process waters that have been involved in or impacted by industry are stored and managed in holding ponds and settling basins, and prevented from being released into the natural water bodies of the region by site operators. This large and growing inventory of on-site water is collectively referred to as oil sands process-affected waters (OSPW) (MacKinnon, 1989). Industrial operations at the AOS have resulted in the accumulation of substantial volumes of OSPW exceeding one billion m^3 (Zubot, 2010).

Although OSPW can contain a mixture of organic and inorganic constituents, the acute and chronic aquatic toxicity of OSPW has been primarily attributed to a relatively persistent group of highly soluble low molecular weight carboxylic acids, known as naphthenic acids (NAs) (MacKinnon and Boerger, 1986; Headley and McMartin, 2004; Quagraine et al., 2005b). Naphthenic acids are a complex mixture of non-cyclic and mono- and poly-cyclic alkanes containing a carboxylic group. They can be characterized by the general empirical formula

$C_nH_{2n+z}O_2$, where *n* indicates the carbon number, and *Z* represents the homologous group series number (i.e., the number of hydrogen atoms that are lost as the structures become more compact or as the fused-ring number increases) (Lai et al., 1996; CEATAG, 1998; Schramm et al., 2000). For example, a *Z* value of 0 would represent the acyclic homologues, and each change in the *Z* value by -2 represents the presence of a ringed structure where -2 would indicate NAs with one ring, -4, two rings, -6, three rings, etc. Moreover, cyclic NAs are also believed to be substituted with alkyl groups, usually consisting of a small methyl or ethyl group, denoted by the letter *R* (CEATAG, 1998).

Since operations began at the AOS over 40 years ago, no off-site release of OSPW has been approved (Griffiths et al., 2006). As a result of the industry's "zero discharge" policy (Quagraine et al., 2005b), OSPW is currently stored on-site in designated retention ponds for recycling and re-use in operations, and future reclamation. Prior to its return to the natural environment, OSPW must meet an acceptable water quality that will ensure that there are no detrimental effects on the receiving environments. Both active and passive remediation processes are being investigated to help improve water quality. Passive treatment of OSPW using natural bioremediation for the removal of dissolved organics and associated toxic properties has focused on the application of biotic processes in wetland and lake habitats. However, there are currently no ambient fresh or marine water quality guidelines for NAs in Canada or the United States. This is primarily due to the lack of sufficient chronic toxicity data regarding the long-term exposure of aquatic biota to NAs (CEATAG, 1998). Therefore, "performance" based wetlands and wet landscape reclamation options are currently being investigated at the AOS. In this strategy, OSPW would be directed through constructed or already existing wetlands, as well as lake systems (e.g., end pit lakes) for the mitigation of undesirable OSPW properties, including the removal of NAs. Reduction or alteration of NAs concentrations and profiles in OSPW so that the associated aquatic toxicity is reduced, could in principle allow the OSPW to be released from reclamation sites (e.g., wetlands, lakes, etc.) into the regional aquatic environment.

One important potential role of wetlands or wet landscapes in oil sands reclamation is to provide sufficient time, biota, and environmental conditions to stimulate NAs biodegradation and to

reduce the associated OSPW aquatic toxicity. Under optimum conditions, NAs are subject to degradation by indigenous microbial communities that have been identified as naphthenate-degraders (Foght et al., 1985; Herman et al., 1994b; Del Rio et al., 2006). Previous studies have shown that when isolated from the input of "fresh' OSPW, aquatic reclamation environments maintained under natural climatic conditions of the AOS region are capable of reducing the total concentration of NAs (Holowenko et al., 2002; Quagraine et al., 2005a). However, evidence suggests that the lower molecular weight and the least cyclic NAs undergo more rapid biodegradation, but the remaining recalcitrant fraction of NAs have half-lives of 12 to 14 years (Han et al., 2009).

This study attempted to describe and quantify the selective biodegradation of NAs congeners and correlate the observed changes in total NAs concentration and composition (i.e., NAs fingerprint profile) with changes, or lack thereof, in the aquatic toxicity of OSPW. For the first time, the relationship between the structure and persistence of NAs mixtures and the associated toxicity is described for OSPW contained in a simulated wetland environment with continual recharge. The present work takes the research conducted in Chapter 4 a step closer towards understanding the effectiveness of natural bioremediation strategies that are proposed to facilitate the aquatic reclamation of OSPW at the AOS.

5.3 Materials and Methods

5.3.1 Experimental Design and Wetland Microcosm Exposure

Two sources of OSPW from Syncrude Canada Ltd. (Syncrude) and Suncor Energy Inc. (Suncor); commercially available Refined Merichem naphthenic acids (Merichem NAs) (Merichem Chemicals & Refinery Services LLC, Houston, TX) dissolved in municipal tap water (positive controls); and municipal tap water (negative controls) were used to charge and recharge 20.8-L flow-through, simulated wetland microcosms in a controlled-environment chamber at the Toxicology Centre, University of Saskatchewan (Saskatoon, SK). Sediment collected from a natural, non-oil sands impacted wetland was added as a 3-cm layer on the bottom of each microcosm. Laboratory microcosms were constructed to simulate a simplistic natural wetland habitat similar to those that could be used in the reclamation of OSPW at the AOS. An array of indigenous microbial communities present in the input OSPW and in the natural sediments (both

added to the microcosms) were the main sources of microbial activity in each system. Overall, there were 12 different treatment scenarios, tested in triplicate, resulting in a total of 36 microcosms.

Details of this original experiment are presented in section 4.3.1 of Chapter 4. However, in this part of the study only the microcosms maintained under the long hydraulic retention times (400 days) were investigated for selective NAs degradation, as OSPWs maintained under these long HRTs were associated with the largest reductions in total NAs concentrations. It is also noted that since nutrient addition was previously found to have no effect on the degradation of NAs in the treatments maintained under long HRTs (400-day) (Chapter 4), all replicates within the same water type, with and without nutrient additions, were pooled such that each treatment water type now had a total of six replicates. The resulting consolidated experimental matrix is shown in Figure 5.1.

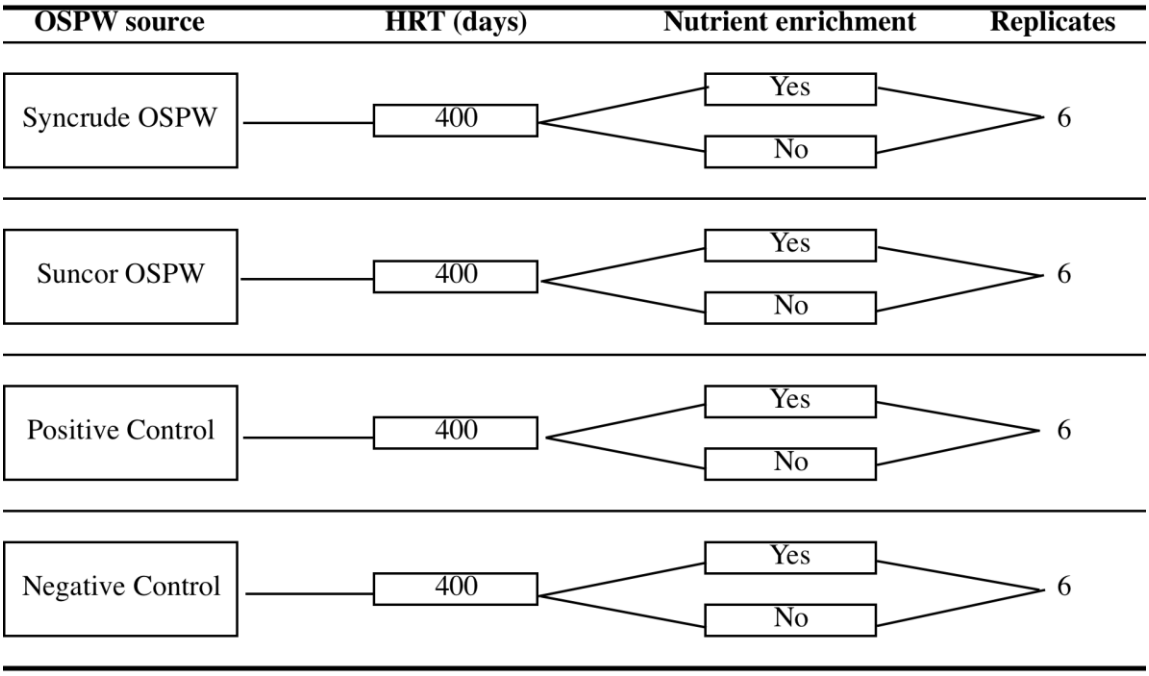


Figure 5.1. Experimental matrix for the 52-week, simulated wetland laboratory microcosm study with oil sands process-affected waters (OSPW) maintained under a 400-day hydraulic retention time. Nutrient enrichment (5 mg/L $NaNO_3$ and 0.64 mg/L Na_2HPO_4) had no effect on the degradation of naphthenic acids and thus the microcosms with and without nutrients were pooled such that each treatment water type had a total of six replicates.

5.3.2 Naphthenic Acids Analysis

Output water samples from all individual microcosms (post-treatment) were collected at time = 0, 16, 36, and 52 weeks and stored at 4°C until analysis. A subset of these samples were stored for subsequent toxicity assessment using the Microtox® bioassay (see ahead; section 5.3.5). Naphthenic acids analysis for characterization of parent constituents of the empirical formula $C_nH_{2n+z}O_2$ and its hydroxylated metabolites of the empirical formula $C_nH_{2n+z}O_3$ was conducted using high performance liquid chromatography/quadrupole time of flight-mass spectrometry (HPLC/QTOF-MS) as described in Bataineh et al. (2006) at the Department of Laboratory Medicine and Pathology (University of Alberta, Edmonton, AB). "Fingerprint" profiles, or distributions of groups of NAs congeners, based on carbon number, *n*, and *Z* series were produced. Negative control test waters were not analyzed for NAs using HPLC/QTOF-MS because the total NAs concentrations were negligible as measured by Fourier transform infrared (FTIR) spectroscopy (Chapter 4).

Due to logistic constraints, the NAs fingerprint profile analysis was not performed on each of the six replicate samples. Instead, at each time interval (0, 16, 36, and 52 weeks), 1-ml from the first three replicates was combined to produce a 3-ml composite sample. Next, 1-ml from the remaining three replicates was combined to produce a second 3-ml composite sample. As a result, two composite samples for both Syncrude and Suncor OSPW treatment waters were subsequently analyzed by HPLC/QTOF-MS to produce two NAs fingerprints each. These two NAs fingerprints were averaged to produce one NAs fingerprint for each of the OSPW treatment water types for each of the four time intervals. The average relative percent difference (based on the total abundance of NAs congeners detected) between the NAs fingerprints combined at each of the four time intervals was 12.3 ± 2.7% (mean ± SE; n =2). Taking a composite of the replicates and averaging the two NAs fingerprints reduced within-treatment variability, even though it remained un-quantified. In the end, four NAs fingerprints, one for each of the four time intervals, were produced for each treatment water type.

To produce a NAs fingerprint profile, each of the composite samples were first filtered through 0.45-µm Millex® syringe filters (Millipore, Billerica, MA) and then diluted with methanol to achieve a final solution containing 60% (v/v) methanol. An internal standard, tetradecanoic

acid-1-^{13}C ($C_{14}H_{28}O_2$), was added to each sample to correct for changes in instrumental sensitivity and retention time drift. Chromatographic separation was performed using a micro-HPLC system (Series 1100, Agilent Technologies, Waldbronn, Germany) and an Aquasil reverse-phase capillary column (150 mm x 0.5 mm, 3 μm) (Thermo Fischer Scientific Inc., Waltham, MA). Injection volumes were 3 μl. The mobile phases included eluant A; 10 mM ammonium acetate and eluant B; methanol (0.1% v/v acetic acid). The column flow rate was 10 μl/min and a gradient elution program was used as follows: 0 to 5 min 60% (v/v) B, ramped to 70% (v/v) B by 7 min, ramped to 100% (v/v) B by 25 min, and held for 5 min before returning to initial conditions and equilibrating for 10 min prior to injection of the next sample.

Detection was performed in full-scan (*m/z* 80 to 500 at rate of 1 scan/s) on an API QSTAR Pulsar i mass spectrometer (Applied Biosystem/MDS Sciex, Concord, ON) equipped with an ion spray source and running Analyst QS 1.1 software (Applied Biosystem, Foster City, CA). The system was operated in negative ion mode, and the source and inlet parameters were optimized by infusion of analytes at 10 μl/min. Optimized parameters were: ion spray capillary voltage of -4300 V, curtain gas flow rate of 1.13 L/min, nitrogen nebulizer gas flow rate of 4 L/min, DP1 -35 V, DP2 -15 V, and a focusing potential of -200 V. Mass calibration was performed with model NA compounds (142 to 360 Da) (Sigma-Aldrich Canada, Oakville, ON).

Tetradecanoic acid-1-^{13}C ($C_{14}H_{28}O_2$) was used as an internal standard to quantify NAs in each sample by relative response. A 50 μl volume of internal standard stock solution (10 mg/L) was added to 950 μl of sample for HPLC/QTOF-MS analysis. The relative response of specific NAs isomer classes (i.e., for each *Z* and *n* combination), relative to an internal standard, was calculated for each sample to determine quantitative changes among samples and over time. The total NAs response was based on the summed response of all detected NAs. Absolute quantification was not possible using the NAs fingerprint method because there were no appropriate standards for such complex mixtures. However, this method is internally quantitative, such that the relative concentration changes can be determined based on the relative response to the internal standard for each NAs isomer classes (i.e., for each *Z* and *n* combination). Validation of this method is described in greater detail in Bataineh et al. (2006).

5.3.3 Biodegradation Kinetics

The biodegradation of NAs was fit to a first-order degradation kinetic model. Using the NAs fingerprint profiles, the changes in the fractional abundance of NAs congeners were fit to a first-order kinetic model described by the integrated form of Equation 5.1,

$$\ln(C/Co) = kt \qquad \text{(Eqn. 5.1)}$$

where C is the percent abundance at time t, standardized to time zero, Co is the initial percent abundance at time zero, k is the reaction rate coefficient (slope), and t is time in weeks. The distributions of the fractional abundance of NAs congeners (i.e., NAs fingerprint) at time zero were set to equal 100% (i.e., Co). The decrease in the abundance at the subsequent time points were standardized to this initial value and indicated the amount remaining in percent (i.e., C).

First-order biodegradation kinetics were obtained by plotting the negative of the natural log of the C/Co ratio versus time for select groups of NAs isomers. Based on the concept of least squares, a linear regression analysis was used to calculate the equation for the "best fit" line from the four time points. The slope of the line was used as an estimate of the reaction rate coefficient, k. Half-lives were calculated using Equation 5.2. SigmaPlot® software (Ver 10.0 Systat Software Inc.) was used to plot all multiple linear regressions.

$$t_{½} = 0.693/k \qquad \text{(Eqn. 5.2)}$$

5.3.4 Naphthenic Acids Degradation Based on *Z* Series, Carbon Number, and Clusters

To describe the selective biodegradation of NAs congeners, the first-order regression kinetics for select NAs isomer groups were calculated by using the average NAs fingerprint profile data for each OSPW type. Total NAs biodegradation was quantified by measuring the change in the total abundance in the NAs fingerprints over the four time intervals for Syncrude and Suncor OSPW separately. In addition, changes in the NAs fingerprint profile during the 52-week study period were examined according to Z series (Z = -2, -4, -6, -8, -10, and -12), carbon number, n (n = 11 to 20), and arbitrarily defined groups (i.e., clusters). Naphthenic acids with a Z series of 0 were not detected using the HPLC/QTOF-MS analysis of both Syncrude and Suncor OSPWs, and

therefore were not included in this assessment. Investigation of the selective biodegradation of Merichem NAs in the positive control treatment was not possible because of their rapid degradation.

The fractional abundance of NAs isomers measured in the average NAs fingerprint profile was summed for each *Z* series of -2, -4, -6, -8, -10, and -12 across all carbon numbers. For example, the fractional abundance for *Z* series -2 was the sum of the response measured for *Z* series = -2 at carbon numbers 11 through 20. The total NAs fingerprint profile was thus divided into six sections. All six regression lines representing each *Z* series were analyzed and compared statistically. Similarly, the fractional abundance of NAs isomers measured in the average NAs fingerprint was summed for each carbon number, 11 to 20, across all *Z* series. For example, the fractional abundance for carbon number 11 was the sum of the response measured for carbon number 11 at *Z* series -2, -4, -6, -8, -10, and -12. In this case, the total NAs fingerprint profile was divided into 10 sections. All 10 regression lines representing each carbon number were analyzed and compared statistically.

The effects of carbon number, *n*, and *Z* series on the biodegradation rate of NAs were not independent. Therefore, the rate of biodegradation of the individual peaks (i.e., both the *n* and *Z* series effects combined) was investigated. For ease of interpretation, the numerous peaks were grouped into nine sub-classes labelled as clusters A to I (Figure 5.2). The fractional abundance of NAs isomers measured in the average NAs fingerprint was summed for each of the nine clusters. For example, the fractional abundance for cluster A was the sum of all responses detected for carbon numbers 11 to 13 and *Z* series -2 and -4. Seven out of the nine regression lines, each representing a cluster, were analyzed, plotted, and compared statistically. Clusters C and G were not included in the analysis because no response was detected within these sub-classes using HPLC/QTOF-MS.

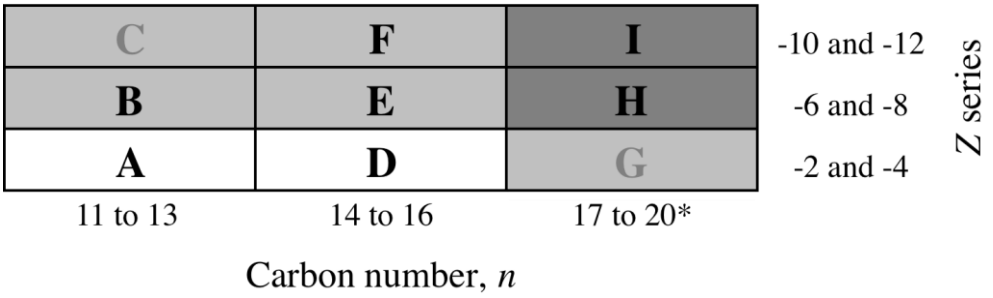


Figure 5.2. A model naphthenic acids (NAs) fingerprint arbitrarily divided into nine clusters labelled A to I. The darkest shaded regions are associated with the most persistent NAs congeners. The boxes without shading represent NAs congeners with the highest rates of biodegradation. This pattern was identical for both Syncrude and Suncor OSPW treatments. No NAs were detected in clusters C and G for either OSPW treatments, and are therefore labelled in grey. *No NAs were detected at carbon number 20 in Suncor OSPW.

An initial analysis of covariance based on the *F* test was used to identify a significant difference between the slopes of the regression lines (Zar, 1999). Subsequent Tukey pairwise comparisons between each pair of slopes were used to determine which slopes were significantly different from each other (Zar, 1999). All calculations were performed using Microsoft® Excel (2002) and with a 95% (α =0.05) level of confidence.

5.3.5 Microtox® Bioassay

Toxicological bioassays were performed at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK) on each of the replicate samples that were combined for the NAs fingerprint profile analysis at exposure times of 0, 16, 36, and 52 weeks in the wetland microcosms. The Microtox® M500 Toxicity Analyzer (Azur Environmental, formerly Microbics Corporation, Carlsbad, CA) using the bioluminescent bacteria *Vibrio fischeri* and related computer software (Microtox® Ver 7.11) (Azur Environmental, 1989) was used to assess the toxicity of the treatment waters. The 15 minute, 100% Basic Test protocol was used to estimate the EC_{20} of each sample. Naphthenic acids are weak organic acids and thus the degree of proton dissociation and solubility depends upon their pK_a and the ambient pH of the surrounding medium. To determine the toxicity without the influence of pH, the pH of the samples was adjusted to between 7 and 8 using 0.25 N HCl or 1 % (v/v) NaOH prior to analysis. This adjustment

stabilized the NAs protonated/de-protonated equilibrium and allowed the *V. fischeri* bacteria to respond under optimum conditions as described in the Microtox® manual, A Toxicity Testing Handbook (Azur Environmental, 1989). Furthermore, two reference toxicants, phenol and zinc sulfate, were analyzed to verify the performance of the Microtox® system. All pH adjustments, and testing of reference toxicants and samples, were completed according to the manufacturer's protocol (Azur Environmental, 1989). The measured phenol and zinc sulfate EC_{50} values were within their recommended ranges of 13 to 26 mg/L and 3 to 10 mg/L, respectively. Supplies for the Microtox® bioassay were purchased from Osprey Scientific (Edmonton, AB).

Linear regression analyses of Microtox® EC_{20} values and wetland exposure time, and the total NAs abundance and wetland exposure time, were completed for both Syncrude and Suncor OSPW post-treatment output waters. The slopes of the regression lines were tested to see if they were significantly different from zero (i.e., was the toxicity and the total NAs abundance changing over time?) according to the methods for analysis of variance, based on the *F* test (Zar, 1999). The regression lines for the Microtox® EC_{20} and wetland exposure time, and the total NAs abundance and wetland exposure time, were plotted together using SigmaPlot® software (Ver 10.0 Systat Software Inc.).

5.4 Results

5.4.1 Selective Naphthenic Acids Biodegradation

The individual peaks in a NAs fingerprint profile represent the fractional abundance of NAs compounds that are classified together on the basis of a single carbon number, *n*, and *Z* series. However, the actual number of individual compounds that are present in each group is unknown. Naphthenic acids biodegradation kinetics can be estimated by quantifying changes in each peak of the NAs fingerprint profile over exposure time in the wetland microcosms. Using this method, the influence of *Z* series, carbon number (*n*), and the arbitrarily defined clusters (A to I) on NAs biodegradation rates were investigated. A qualitative assessment of the NAs profiles for Syncrude and Suncor OSPW, at four time points revealed a structure-persistence relationship after a 52-week exposure period to the simulated wetland microcosms. Conversely, the commercial Merichem NAs in the positive control treatment waters were almost completely removed after 16 weeks (Figure 5.3).

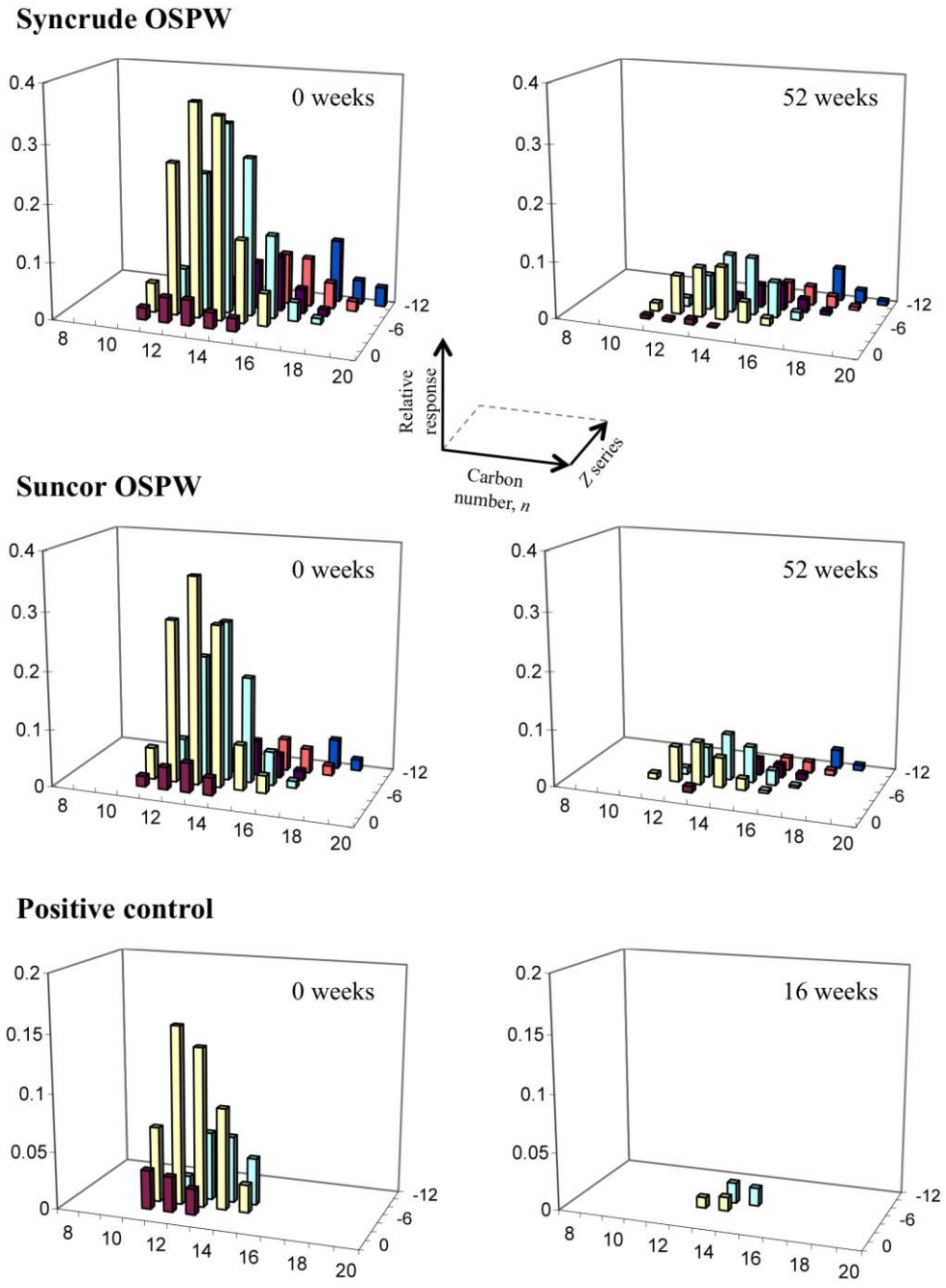
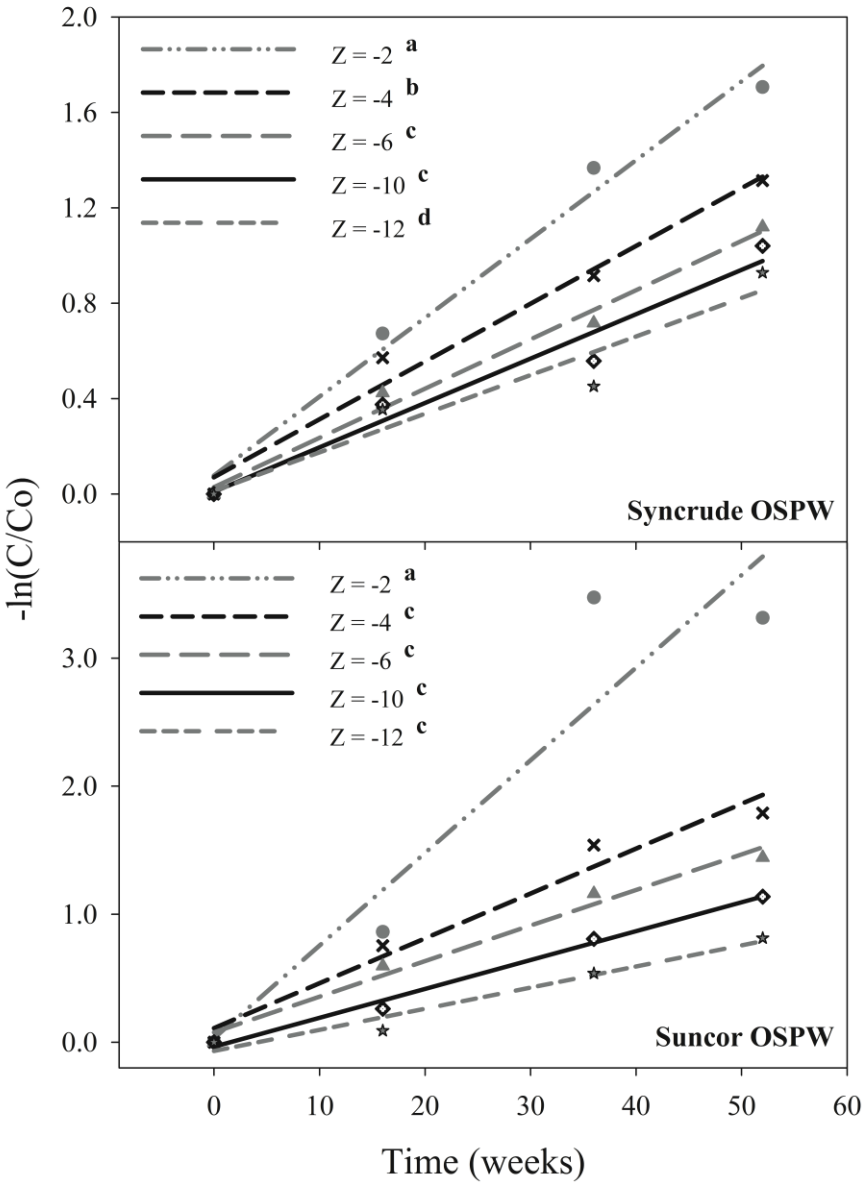


Figure 5.3. Representative naphthenic acids (NAs) fingerprints for Syncrude OSPW, Suncor OSPW, and the positive control treatment water showing changes in the fractional abundance of NAs homologues after exposure to a simulated wetland environment for 52 weeks.

The effect of *Z* series (i.e., cyclicity) on NAs biodegradation rates revealed a strong trend that was consistent between both Syncrude and Suncor OSPW. Naphthenic acids with higher negative *Z* series numbers, or higher degrees of cyclization, were more persistent than NAs with lower, less negative *Z* series numbers (Figure 5.4 and Table 5.1). Furthermore, NAs with higher carbon numbers ($n \geq 16$) were more persistent than those with lower carbon numbers ($n \leq 15$), and again, this trend was consistent between both Syncrude OSPW and Suncor OSPW NAs (Figure 5.5 and Table 5.2).



[a] Significantly different from *Z* series -4 to -12

[b] Significantly different from *Z* series -2, -8, and -12

[c] Significantly different from *Z* series -2

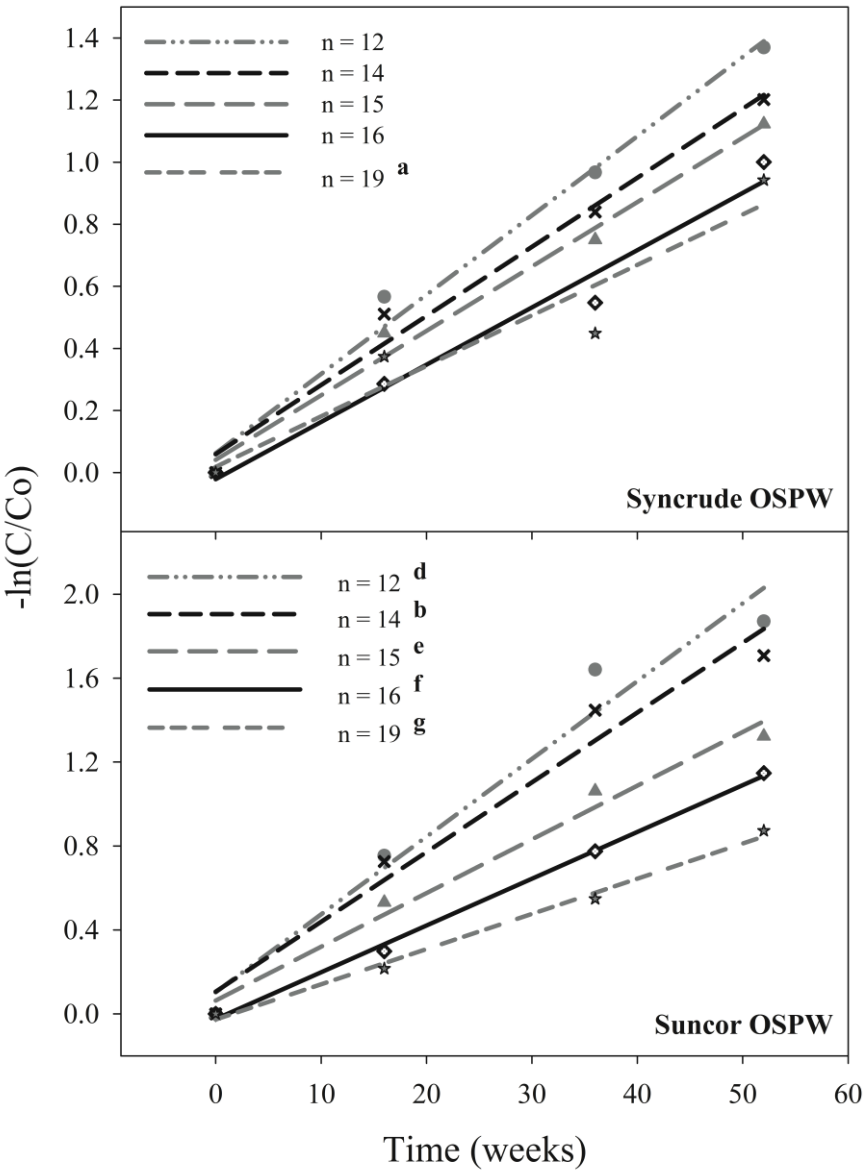
[d] Significantly different from *Z* series -2 and -4

Figure 5.4. Effect of *Z* series on the rate of biodegradation of naphthenic acids in Syncrude and Suncor OSPW. [a-d]Denotes a significant difference between slopes as determined by analysis of covariance and Tukey pairwise comparisons (P <0.05); n =4.

Table 5.1. Naphthenic acids biodegradation kinetics by *Z* series for Syncrude and Suncor oil sands process-affected waters.

Z series	Slope	NAs half-life (weeks)	r^2	Significantly different (P <0.05)	% Reduction after 52 weeks
Syncrude NAs					
Z = -2	0.0330	21	0.9835	[a]different from -4 to -12	82
Z = -4	0.0242	29	0.9797	[b]different from -2, -8, & -12	73
Z = -6	0.0206	34	0.9874	[c]different from -2	67
Z = -8	0.0160	43	0.9648	[d]different from -2 & -4	59
Z = -10	0.0186	37	0.9580	[c]different from -2	65
Z = -12	0.0162	43	0.9246	[d]different from -2 & -4	60
Suncor NAs					
Z = -2	0.0724	10	0.8865	[a]different from -4 to -12	96
Z = -4	0.0350	20	0.9656	[c]different from -2	83
Z = -6	0.0278	25	0.9798	[c]different from -2	76
Z = -8	0.0189	37	0.9738	[c]different from -2	64
Z = -10	0.0226	31	0.9920	[c]different from -2	68
Z = -12	0.0165	42	0.9623	[c]different from -2	56

[a-d]Denotes a significant difference between certain slopes as determined by analysis of covariance and Tukey pairwise comparisons (P <0.05); n =4.



[a] Significantly different from carbon number 20

[b] Significantly different from carbon numbers 17 to 19

[d] Significantly different from carbn numbers 16 to 19

[e] Significantly different from carbon number 11

[f] Significantly different from carbon numbers 11 and 12

[g] Significantly different from carbon numbers 11 to 14

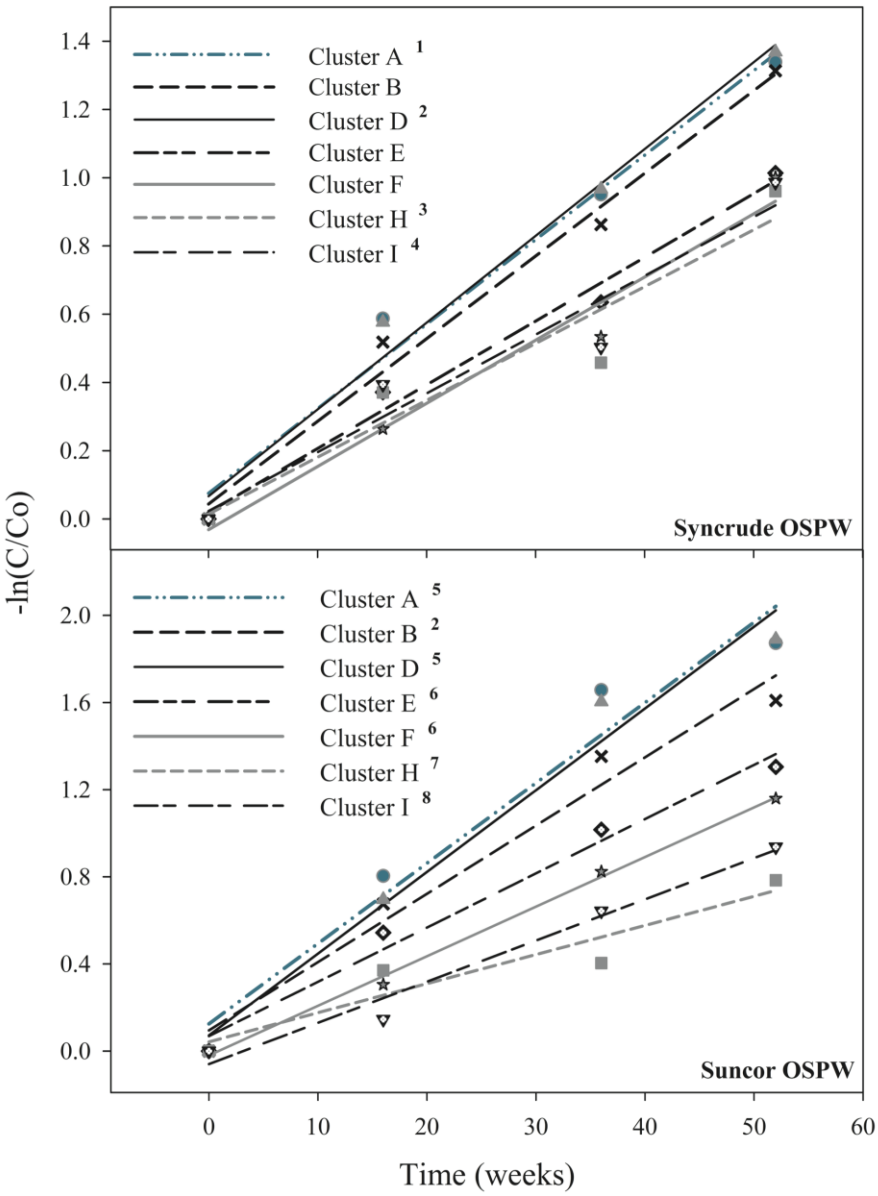
Figure 5.5. Effect of carbon number, *n*, on the rate of biodegradation of naphthenic acids in Syncrude and Suncor OSPW. [a, b, d-g]Denotes a significant difference between slopes as determined by analysis of covariance and Tukey pairwise comparisons (P <0.05); n =4.

Table 5.2. Naphthenic acids biodegradation kinetics by carbon number, *n*, for Syncrude and Suncor oil sands process-affected waters.

Carbon number	Slope	NAs half-life (weeks)	r^2	Significantly different (P <0.05)	% Reduction after 52 weeks
Syncrude NAs					
n = 11	0.0218	32	0.9973	—	68
n = 12	0.0255	27	0.9863	—	75
n = 13	0.0244	28	0.9756	—	74
n = 14	0.0223	31	0.9831	—	70
n = 15	0.0208	33	0.9868	—	67
n = 16	0.0184	38	0.9749	—	63
n = 17	0.0165	42	0.9027	[a]different from 20	62
n = 18	0.0167	42	0.9399	[a]different from 20	61
n = 19	0.0163	43	0.9120	[a]different from 20	61
n = 20	0.0268	26	0.9348	[b]different from 17-19	78
Suncor NAs					
n = 11	0.0446	16	0.9579	[c]different from 15-19	89
n = 12	0.0371	19	0.9635	[d]different from 16-19	85
n = 13	0.0334	21	0.9590	[b]different from 17-19	82
n = 14	0.0332	21	0.9684	[b]different from 17-19	82
n = 15	0.0256	27	0.9822	[e]different from 11	73
n = 16	0.0223	31	0.9975	[f]different from 11 & 12	68
n = 17	0.0172	40	0.9923	[g]different from 11-14	60
n = 18	0.0188	37	0.9521	[g]different from 11-14	60
n = 19	0.0168	41	0.9933	[g]different from 11-14	58

[a-g]Denotes a significant difference between certain slopes as determined by analysis of covariance and Tukey pairwise comparisons (P <0.05); n =4.

The integrated effect of carbon number, *n*, and *Z* series on the rate of NAs biodegradation was investigated by examining the percent reduction of individual peaks that were arbitrarily grouped into nine clusters. Clusters H and I, representing NAs congeners with carbon numbers 17 to 20 and *Z* series -6 to -12, were the most persistent. In contrast, clusters A and D, representing NAs congeners with carbon numbers 11 to 16 and *Z* series -2 to -4, degraded the fastest (Figure 5.6). Although significant differences ($P < 0.05$) were only detected between some slopes, the preferential order of cluster biodegradation was identical for both Syncrude and Suncor OSPW. The most persistent clusters were associated with areas of the NAs fingerprint that constitute the most complex NAs compounds with the most carbon numbers and number of rings as highlighted in a model NAs fingerprint (Figure 5.2). A comparison of NAs biodegradation kinetics for clusters A to I for both Syncrude and Suncor OSPW are presented in Table 5.3.



[1] Significantly different from Cluster H
[2] Significantly different from Clusters H and I
[3] Significantly differenttfrom Clusters A and D
[4] Significantly different from Cluster D
[5] Significantly different from Clusters E to I
[6] Significantly different from Clusters A, D, and H
[7] Significantly different from Clusters A to F
[8] Significantly different from Clusters A to D

Figure 5.6. Comparison of biodegradation rates between the arbitrarily defined clusters (A to I) of naphthenic acids in Syncrude and Suncor OSPW. [1-8]Denotes a significant difference between slopes as determined by analysis of covariance and Tukey pairwise comparisons (P <0.05); n =4.

Table 5.3. Biodegradation kinetics for the arbitrarily defined naphthenic acids clusters (A to I) in Syncrude and Suncor oil sands process-affected waters.

Cluster	Slope	NAs half-life (weeks)	r^2	Significantly different (P <0.05)	% Reduction after 52 weeks
Syncrude NAs					
A	0.0248	28	0.9795	[1]different from H	74
B	0.0242	29	0.9866	—	73
D	0.0254	27	0.9846	[2]different from H & I	75
E	0.0187	37	0.9873	—	64
F	0.0185	38	0.9707	—	63
H	0.0167	42	0.9169	[3]different from A & D	62
I	0.0172	40	0.9317	[4]different from D	63
Suncor NAs					
A	0.0369	19	0.9573	[5]different from E to I	85
B	0.0313	22	0.9711	[2]different from H & I	80
D	0.0375	19	0.9750	[5]different from E to I	85
E	0.0249	28	0.9830	[6]different from A, D, & H	73
F	0.0228	30	0.9969	[6]different from A, D, & H	69
H	0.0134	52	0.8985	[7]different from A to F	54
I	0.0189	37	0.9760	[8]different from A to D	61

[1-8]Denotes a significant difference between certain slopes as determined by analysis of covariance and Tukey pairwise comparisons (P <0.05); n =4.

5.4.2 Microtox® Toxicity

A significant decrease (i.e., the slopes of the total NAs concentration regression lines were significantly different from zero) in the total fractional abundance of NAs present in both Syncrude and Suncor OSPW was observed after the 52-week exposure period in the simulated wetland microcosms. However, this decrease in total NAs concentrations was not associated with a decrease in Microtox® toxicity (EC_{20}) (i.e., slopes of the EC_{20} regression lines were not significantly different from zero) for either Syncrude or Suncor OSPW (Figure 5.7). On average, the toxicity (mean EC_{20} ± SE; n =4) of Syncrude OSPW (EC_{20} = 27 ± 5%) was greater than that of Suncor OSPW (EC_{20} = 43 ± 10%).

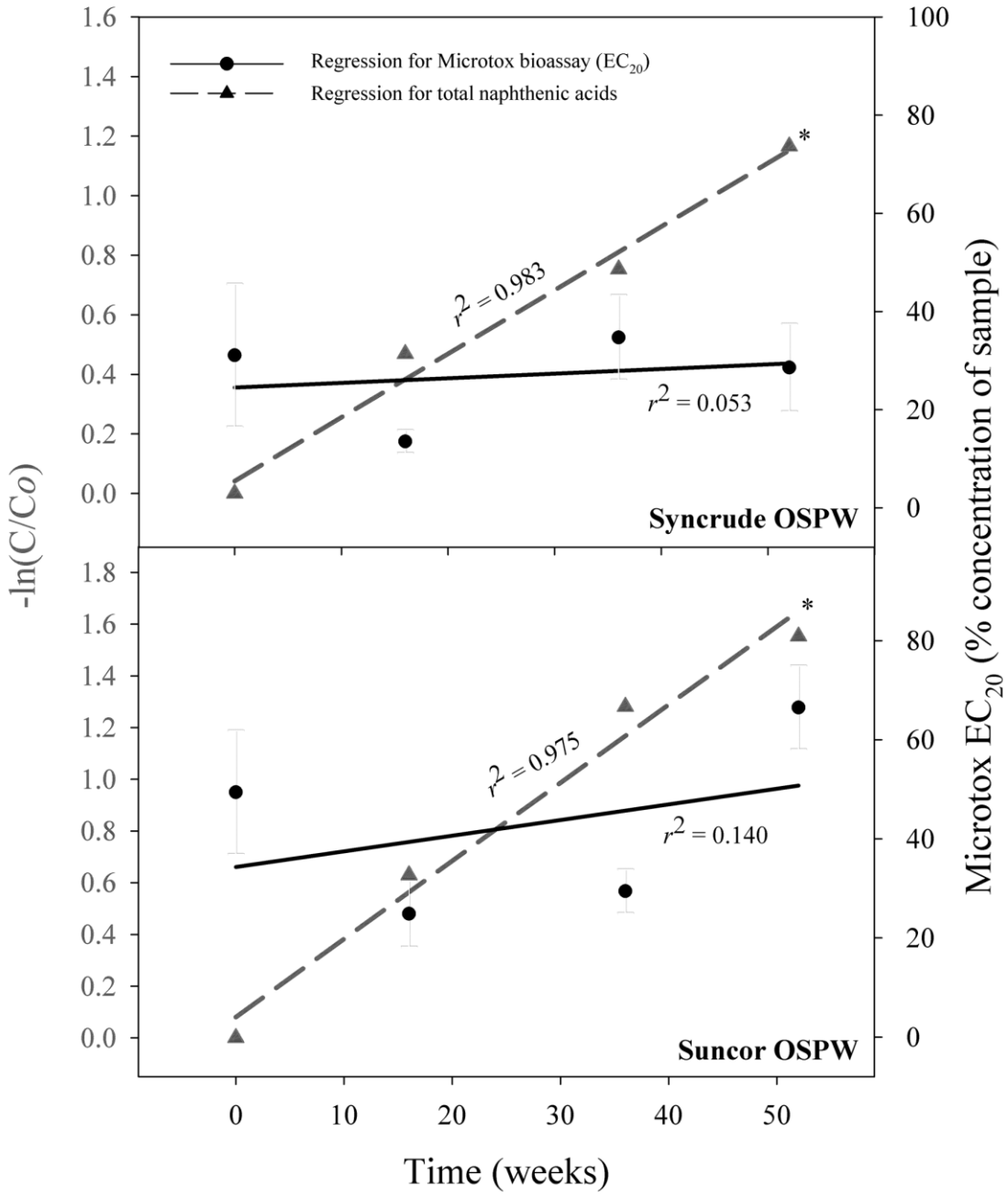


Figure 5.7. Regressions of total naphthenic acids (NAs) degradation versus time, and Microtox® EC_{20} values versus time, for Syncrude and Suncor OSPW. *Denotes a slope that was significantly different from zero (P <0.05; n = 4) based on an analysis of variance (ANOVA) of slopes. The Microtox® EC_{20} slopes were *not* significantly different from zero (P >0.05; n = 4).

5.5 Discussion

The inverse relationship between the number of rings in a NAs compound and the biodegradation rate observed in this present study is consistent with the findings from previous studies using both model NAs compounds (Lai et al., 1996; Del Rio et al., 2006) and OSPW NAs (Han et al., 2008). In the past, NAs with lower carbon numbers were found to be more susceptible to biodegradation (Clemente et al., 2004). For example, the microbial degradation of a model single ring NA with 6 carbons (cyclopentane carboxylic acid) was faster than the microbial degradation of a NA with 7 carbons (cyclohexane carboxylic acid) (Herman et al., 1993). Conversely, Han et al. (2008) used high-resolution mass spectrometry to show that carbon number had little effect on the rate of OSPW NAs biodegradation by examining the HPLC/QTOF-MS response of carbon number *within* each *Z* series. This lack of a carbon number effect may be due to the slightly different method used to analyze the NAs fingerprint data by Han et al. (2008) than that used in the present study where the response of carbon number was examined *across* all *Z* series.

Recent studies conducted on NAs mixtures using high resolution HPLC/QTOF-MS suggest that biodegradation rates of NAs are associated with the number and size of alkyl side chains (alkyl branching), the number of carbons (carbon number, *n*), and the actual number of rings (*Z* series) (Bataineh et al., 2006; Han et al., 2008). Han et al. (2008) determined that increasing cyclization (i.e., more negative *Z* series) decreased the biodegradation rates of NAs. In addition, they investigated the effect of carbon number, *n*, on the biodegradation rates *within* a *Z* series. This eliminated the influence of the number of rings, but still allowed for the possibility that the observed effects might be confounded by the degree of alkyl branching due to the inherent relationship between the number and size of alkyl side chains and carbon number. In fact, Bataineh at al. (2006) have presented evidence that the extent of alkyl branching may explain the decreased biodegradation rates of recalcitrant fractions of NAs mixtures. The lack of a carbon number effect *within* a *Z* series observed by Han et al. (2008) combined with the results from Bataineh et al. (2006) suggests that an increase in alkyl branching and cyclization have more to do with the observed decrease in biodegradation rates than carbon number.

In view of that, the decrease in biodegradation rates relative to higher carbon numbers, investigated by summing the responses *across* all *Z* series in our present study, is likely associated with the actual number of rings (due to the increase in the relative abundance of NAs congeners with increasing number of rings for each higher carbon number), and possibly the number and size of alkyl side chains (i.e., alkyl branching). Therefore, *Z* series had more of an influence on the NAs biodegradation rate than carbon number, which was consistent with the conclusion drawn by Han et al. (2008). Moreover, the observed selective biodegradation of NAs based on *Z* series was consistent with the structure-persistence relationship observed and described to be a signature of NAs biodegradation (Han et al., 2008).

The inability to detect significant differences between many of the estimated slopes, highlighted how closely related the different NAs congeners are and the analytical challenge that exists when studying the fate of complex NAs mixtures. Differences between closely related NA homologues might not be detected when examining the kinetics of the individual peaks in a given NAs fingerprint profile. For this reason, and for ease of interpretation, the integrated effect of carbon number and *Z* series on the rate of NAs biodegradation was investigated by examining the percent reduction of peaks that were arbitrarily grouped into the nine clusters. The overall biodegradation trend, based on the arbitrarily defined clusters, was identical for both Syncrude and Suncor OSPW. This suggested that the microbial communities, the relative degradation rates, and possibly the degradation pathways used to degrade NAs were the same regardless of the source of OSPW.

Using Syncrude OSPW maintained in incubation vials at room temperature, Han et al. (2008) estimated the half-lives (based on first-order kinetics) of OSPW NAs congeners, having carbon numbers between 12 and 17, and *Z* series values of -2 and -4, to be approximately 7 to 14 weeks. These estimates were nearly 0.5 times lower than those determined in the present microcosm study (half-lives between 19 and 28 weeks for both OSPWs) for NAs congeners present in clusters A and D (similar carbon numbers and Z series values). The half-lives of the more complex and persistent NAs congeners with carbon numbers between 15 and 17, and *Z* series values of -6 and -8 (half-lives between approximately 21 and 34 weeks; Han et al. 2008), were also lower than the half-lives (42 and 52 weeks in Syncrude and Suncor OSPW, respectively) of

the persistent NAs congeners present in cluster H. The slightly longer half-lives observed in the present microcosm study are likely the result of the continuous recharge of OSPW and associated replenishment of NAs, as opposed to the isolated incubations with no OSPW or NAs recharge used by Han et al. (2008).

The β-oxidation pathway is the preferred mechanistic route by which aerobic micro-organisms degrade both simple and complex aliphatic carboxylic acids (Taylor and Trudgill, 1978; Quagraine et al., 2005a; Han et al., 2008). Straight chain carboxylic acids are degraded by a sequence of oxidation processes that occur at a carbon that is β to the carboxyl group, forming new carboxylic acids with fewer carbons than the parent compound. The process continues as structures are shortened until the pathway is either arrested or hampered due to the presence of quaternary and tertiary carbons at specific locations in close proximity to the carboxyl group (Quagraine et al., 2005a). The aerobic biodegradation of more complex carboxylic acids (e.g., cycloalkyl carboxylic acids) follows a similar pathway, but is expected to begin first with the β-oxidation of the side chains (Herman et al., 1994b). The biodegradation of the rings is initially limited due to the stability (i.e., lack of terminal carbon) and the relative position of the ring to the carboxyl group, especially if the carboxyl group is attached directly to the ring. Any subsequent branching or proximity of tertiary and quaternary carbon to the β position creates further steric hindrance and impedes biodegradation by this pathway (Han et al., 2008). This would explain why in this present study the biodegradation of the naphthenic acids was slower in more complex homologues with higher degrees of cyclization. The slower biodegradation of rings may be considered as a rate limiting step in the β-oxidation pathway, which would also explain why the increasing carbon number was found to have less of an effect than *Z* series on the persistence of NAs. The hypothesis that biodegradation of NAs is controlled largely by the rate of biodegradation of the rings, regardless of the length of the side chains that are quickly mineralized, is supported by Headley et al. (2002b). In their study, the biodegradation rates of two model NAs that had either a carboxylic acid attached directly to the cyclohexane ring or attached via a methyl group were not significantly different.

The structure-persistence relationship observed in this study is consistent with that described by Holowenko et al. (2002). In their study, the NAs mixtures in aged process waters from the field

contained higher proportions of the C22+ cluster (i.e., heavier molecular weight NAs with the highest carbon numbers and *Z* series). However, in the study presented here, there was no detection of NAs having carbon numbers greater than 20, owing possibly to the different analytical technique used to generate a NAs mixture profile. Holowenko et al. (2002) used low resolution GC-MS to produce the NAs fingerprint profiles. This method has the potential for misclassification of NAs biotransformation products. Oxidized NAs that contain additional oxygen atoms (i.e., hydroxy and dihydroxy NAs) can potentially be mis-labelled as high molecular weight NAs (Clemente et al., 2004; Bataineh et al., 2006). In fact, the biodegradation pathway for cycloalkyl carboxylic acids always lead to the production of hydroxylated intermediates and is considered to be a signature of NAs microbial transformation (Quagraine et al., 2005a; Han et al., 2008). Thus, there is a good possibility that the presence of the highly significant "C22+ cluster" in the NAs fingerprint generated with GC-MS, were artefacts created by false positive detections and misclassifications if hydroxylated NAs were present in the samples being analyzed (Bataineh et al., 2006; Han et al., 2008). In contrast, HPLC/QTOF-MS analysis is a high resolution method that accurately assigns the homologues fitting the $C_nH_{2n+z}O_2$ empirical formula. This will exclude the hydroxylated NAs intermediates from the NAs fingerprints, however, if present these hydroxylated NAs could contribute to toxicity. Nevertheless, both the "C22+ cluster" and the results in this study, using the high resolution method, identified the heavier molecular weight NAs as being the most persistent.

In this research, the concentration and composition of NAs in OSPW, including the concentrations of the selectively persistent clusters, was dependent on the HRT and in turn, would influence the observed residual chronic toxicity, assuming that some other compounds present in OSPW which are not NAs or their metabolites, did not contribute to the observed toxicological response. Given that these microcosms are dynamic, with a continuous recharge of OSPW, the NAs would have been maintained at an approximate steady-state level that may have been sufficient to maintain the toxicity of the microcosm waters. The continual input of "fresh" OSPW, containing relatively more of the lower molecular weight NAs, would allow the microbial community to selectively degrade the lower molecular weight homologues that are more readily biodegradable. This action would lead to an enrichment of the more persistent,

higher molecular weight NAs congeners that would remain after the more labile components of the NAs were utilized as a microbial carbon source.

One assumption in this recharge hypothesis is that the lack of change in the toxic response is related to components in the NAs mixture profile that are more persistent. The concentrations of NAs in clusters H and I decreased by as little as 54 to 62% of their original values, while clusters A and D, the less complex and lower molecular weight NAs, decreased by as much as 75 to 85% (Table 5.3). The relative persistence of clusters H and I, or specific compounds therein, and the persistent chronic toxicity observed indicates that these refractory NAs constituents may be responsible. It is plausible that the no observed effect concentrations (NOECs) for clusters H and I were not reached because the biodegradation data did not suggest that the removal of these compounds peaked during the 52 weeks of the study. Based on the rates of degradation determined over the term of this study, it is possible that the residual chronic toxicity may have been removed after longer wetland exposure when the concentrations of clusters H and I would fall below some unidentified threshold values. Similarly, increasing the HRT might cause the microbial communities to shift over time towards those favouring the degradation of higher molecular weight NAs (clusters H and I), after initial communities utilized the bulk of the readily biodegradable lower molecular weight fractions. However, in a natural wetland in the oil sands region, other sources of organic matter such as detritus may reduce the need to utilize the higher molecular weight NAs fraction as a carbon source. With the much longer residence times that may be obtained in lake environments associated with reclamation options such as end pit lakes or wetlands with very low recharge rates, enhanced rates of degradation of clusters H and I may be achieved, possibly resulting in a stronger correlation between Microtox® toxicity and total NAs concentration.

The findings from this study are consistent with field observations demonstrating that aged OSPW (i.e., >3 years) held in various water bodies with minimal recharge, had lower total NAs concentrations than the wet landscape environments that were “fresh” or less aged (Holowenko et al., 2002; Han et al., 2009). In these field studies, acute toxicity of oil sands tailings water as determined using a battery of bioassays was rapidly reduced by natural processes, so that in a time period of one year the OSPW showed no acute toxicity. However, other studies have

shown that the removal of chronic toxicity can take more than 3 years (MacKinnon and Boerger, 1986; Schramm et al., 2000), which is consistent with the persistent Microtox® toxicity (EC_{20}s) observed in this research. With low or no recharge, synonymous with very long HRTs, microbial communities may become more capable of degrading the H and I clusters to below threshold concentrations, thus reducing the associated chronic toxicity of OSPW. Studies utilizing fractionation of NAs mixtures and the subsequent identification of their associated toxicological thresholds need to be conducted to fully address this hypothesis.

5.6 Conclusions

This research has shown that the observed structure-persistence relationship of NAs was similar regardless of the OSPW source. Even with extended HRTs, NAs with the highest degrees of cyclization and highest carbon numbers were shown to be more resistant to degradation. Although significant NAs biodegradation occurred, where there was a decrease in total NAs concentration, there still appeared to be a persistent chronic toxicity response seen with the Microtox® bioassay. Based on the structural changes of the NAs mixtures observed using the HPLC/QTOF-MS method, this chronic toxicity may be a function of the persistence of heavier molecular weight isomers with n = 17 to 20 and Z = -6 to -12 (clusters H and I), and continual OSPW recharge. Therefore, flowing wetlands with slow continual OSPW recharge may be an effective remediation option if the HRT is sufficiently long to provide enough time to reduce the concentrations of the persistent NAs and the associated chronic aquatic toxicity. Alternatively, self-contained aquatic systems with zero or minimal discharge should provide optimized removal of NAs and meet the industry's need for site reclamation and incorporation into a sustainable water management strategy that will include lakes and wetlands. However, as more information about NAs molecular structure and its influence on biodegradation is obtained, optimization of wet landscape design features in oil sands lease closure reclamation options will be possible.

6.0 SORPTION OF OIL SANDS NAPHTHENIC ACIDS TO A HIGHLY ORGANIC WETLAND SEDIMENT

6.1 Abstract

The Clark hot water extraction process used in the open-pit mining operations at the AOS results in the production of large volumes of oil sands process-affected waters (OSPW). These OSPW contain elevated levels of naphthenic acids (NAs), which are toxic to various aquatic organisms. Due to their toxic nature, OSPW are currently retained on-site in engineered settling ponds as wet landscape reclamation strategies are actively being investigated. The organic carbon content of sediments contained within these reclamation wetland and lake systems can potentially influence the NAs concentrations in the overlying OSPW via abiotic processes such as sorption. This research examined the potential for changes in total NAs concentrations and composition in OSPW on account of sorption to highly organic non-oil sands impacted wetland sediment. Results from a preliminary batch-reactor investigation showed a rapid (<1 day) and significant (P <0.001) reduction in total NAs concentrations in OSPW when agitated with the wetland sediment at a ratio of 2:1 v/v (OSPW:sediment). The total organic carbon content (%) of the wetland sediment was 27.6 ± 0.9% (mean ± SD; n =4) and the pH of OSPW in the batch-reactor test vessels containing both OSPW and wetland sediment was 7.1 ± 0.1 (mean ± SE; n =3). The mean percent reduction in total NAs concentrations in the water of the test batch-reactor vessels from Day 1 to 14 was approximately 67% relative to the mean NAs concentrations in the Control OSPW during the 14-day test period, suggesting a greater influence of sorption on the removal of NAs from solution than previously expected. However, no preferential sorption was observed based on the distribution of NAs congeners with respect to carbon number, Z series, and arbitrarily defined clusters.

6.2 Introduction

Oil sands process-affected waters (OSPW) produced during the commercial extraction of bitumen at the Athabasca oil sands (AOS) are both acutely and chronically toxic to aquatic organisms. This toxicity has largely been attributed to the dissolved organic acids called naphthenic acids (NAs). As result, OSPW is currently stored on-site in large holding ponds and settling basins, but with the understanding that eventually reclamation must be undertaken. One

reclamation strategy under investigation is the use of wetlands or wet landscapes to potentially reduce the total NAs concentrations and the overall associated toxicity of OSPW via biodegradation. The pathways of natural or enhanced degradation (abiotic and biotic) still need to be better understood, but experiments using simulated laboratory wetland microcosms (Chapters 4 and 5) provided greater insight into the potential effectiveness of wetlands for the natural remediation of OSPW.

Microorganisms and nutrients innatley present in OSPW and natural wetland sediments were presumed to be responsible for the observed degradation of NAs in the simulated wetlands study (Chapter 4). The structure-persistence relationship described using the NAs fingerprinting analysis (Chapter 5) provided evidence in support of biological degradation of NAs. However, the possibility of sorptive processes contributing to the overall reduction of NAs in the microcosms was not investigated. Evidence of substantial NAs sorption on sediment is limited as NAs are a weak acid having a pK_a between 5 and 6 (Headley et al., 2002a; Headley et al., 2002b) and thus remain dissolved in the ionized form in the slightly alkaline aquatic environments of the AOS waters. Reportedly, NAs in OSPW have low sorption distribution coefficients (K_d) (e.g., <0.5 mL/g on sand and between 1 and 3 units on different clay substrates; (Schramm et al., 2000). However, Janfada et al. (2006) showed that soils having higher organic carbon content have higher levels of adsorption and that the K_d values for OSPW NAs were as high as 17.8 mL/g in synthetic, high salinity ground water (total ion concentration greater than 2400 mg/L) at a pH of approximately 8. This high K_d value was attributed to a decrease in NAs solubility due to the increased availability of binding sites on the soil (i.e., organic fraction of the soil), and the potential "salting out" effect in the presence of elevated levels of Na^+, Ca^{2+}, Mg^{2+}, and SO_4^{2-} ions (Janfada et al., 2006). In addition, Janfada et al. (2006) found preferential sorption of NAs within the carbon number range of 13 to 17 for all *Z* families. Conversely, a "sorption signature", or preferential sorption of NA homologues, as identified using a GC-MS NA fingerprint analysis, was not observed with a 15% decrease in total NAs concentrations in a laboratory batch reactor sorption study with ground water (Gervais, 2004). It was concluded that any observed changes in the NAs "signature" in the ground water samples were likely due to biodegradation and not sorption. However, the author also noted that the preparation of the NAs stock solution used in the study produced a NAs mixture that was low in high molecular weight

compounds. Naphthenic acids isolated from OSPW have a higher proportion of high molecular weight compounds, which may result in greater sorption (Quagraine et al., 2005b). Different groups of NAs congeners have varying degrees of toxicity and thus preferential sorption of NAs may affect the aquatic toxicity of OSPW.

The objective of this study was to characterize potential changes in the total concentration and composition of NAs in OSPW due to sorption. The materials used (the highly organic wetland sediment and OSPW) were analogous to those used in the previous wetland microcosm study (Chapter 4) for consistency.

6.3 Materials and Methods

6.3.1 Test Materials

Syncrude Canada Ltd. (Syncrude) OSPW was collected in September 20101 at a sand dyke seepage point at the Mildred Lake Settling Basin (MLSB), which lies within Syncrude's Mildred Lake site lease at the AOS. The seepage location is easily accessible for most of the year and continuously discharges process water (~10 years of age) after it has slowly passed through a sand dyke under an anaerobic environment. Highly organic wetland sediment, consisting of the top 10 cm of uppermost live vegetation and sediment was collected from a local non-oil sands impacted wetland (Tower Road) within the AOS region on December 16, 2006. Both materials were shipped to the Toxicology Centre, University of Saskatchewan (Saskatoon, SK) and stored at 4°C in the dark until experimentation.

6.3.2 Experimental Design and Protocol

Twelve, 2-L glass jars were used as batch-reactor vessels. Approximately 800 mL of OSPW were added to each of the six control batch-reactor vessels; no sediment was added (Figure 6.1A). The test batch-reactor vessels contained approximately 800 mL of OSPW and 400 g of homogenized wetland sediment (2:1 v/v, OSPW:sediment ratio) (Figure 6.1B). The batch-reactor vessels were sealed and loaded onto a Model 100, Burrel Scientific Mighty Magnum Wrist Action Shaker (Burrel Scientific, Pittsburgh, PA), which was then placed in an environmental chamber at the Toxicology Centre maintained at 4°C for the duration of the 14-

day experiment (Figure 6.2). Batch-reactor vessels were continuously agitated at 285 oscillations per minute for 14 days.

Subsamples of input OSPW were collected for water chemistry (four replicates) and total naphthenic acids (three replicates) analyses prior to OSPW introduction into the reactor vessels. Subsequently, the shaker was stopped and the samples collected on days 1, 7, and 14 from each of the reactor vessels. Day 7 samples were collected from the same subset of vessels sampled at Day 1 (repeated sampling). All samples were collected into 150-mL glass vials and centrifuged (International Centrifuge Universal Model UV, International Equipment CO., Boston, MA) for 25 minutes at 3200 rpm. Each sample was split into two sub-samples, one for NAs and one for general water chemistry analysis (Figure 6.2).

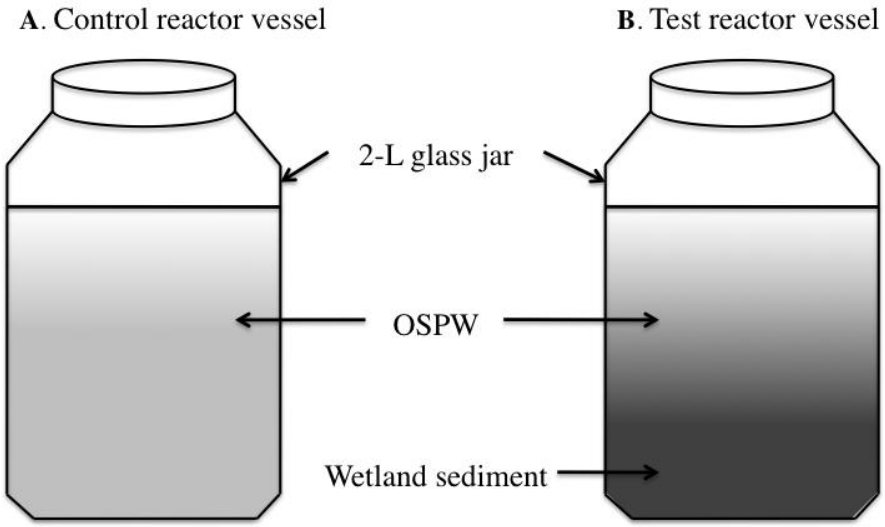


Figure 6.1. Schematic of the batch-reactor vessels for A) controls containing OSPW only, and B) test vessels containing OSPW and wetland sediment.

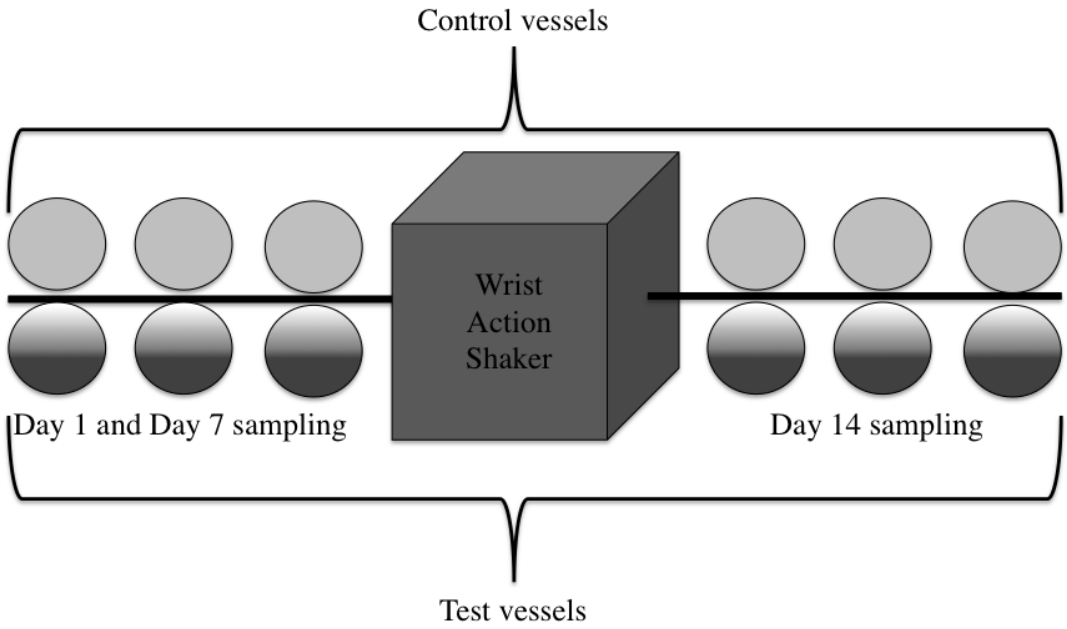


Figure 6.2. Schematic of the control and test batch-reactor vessels positioned on a wrist action shaker for the naphthenic acids sorption experiment.

6.3.3 Water Chemistry, Naphthenic Acids Analysis, and Total Carbon Content of Sediment

General water chemistry measurements were performed at the Toxicology Centre (Saskatoon, SK). Sample water pH was measured using an Orion Model 835 pH meter (Beverly, MA). Alkalinity and total hardness were measured using a HACH Digital Titrator Model 16900 (Hach Company, Loveland, CO). Conductivity was measured with an Orion Model 170 Conductivity Meter (Orion Research Inc., Boston, MA). Total NAs concentrations were measured in all samples using electrospray ionization mass spectrometry (ESI-MS) at Environment Canada (Saskatoon, SK) according to their standard methods (Headley et al., 2002a). Three subsamples of the natural wetland sediment were analyzed for total organic carbon (TOC) by the Department of Soil Science, College of Agriculture and Bioresources, University of Saskatchewan (Saskatoon, SK).

6.3.4 Distribution of Naphthenic Acids Congeners

To assess if selective sorption occurred, the NAs mixture profiles (i.e., distribution of the NAs congeners) were also generated using ESI-MS. However, due to logistical constraints, only a single replicate representing each water type was analyzed (e.g., Input, Control, Day 1, and Day 7). The Day 14 water sample was not assessed due to the similarity of the total NAs concentration on that day to the Day 7 sample where the maximum amount of sorption had already occurred.

In a given NAs mixture profile produced using the ESI-MS method, the fractional abundance of specific NAs isomer classes (relative to an internal standard) is based on each carbon number and *Z* series combination. The distribution of NAs congeners was examined based on carbon number, *Z* series, and arbitrarily defined clusters labelled A through I (see Chapter 5 for a more detailed explanation) to determine quantitative changes among the four samples (e.g., Input, Control, Day 1, and Day 7).

The influence of carbon number on sorption was examined by dividing the NAs mixture profile into 4 groups. The first group contained responses summed for all congeners with carbon numbers C5 to C13. The second group contained NAs congeners from C14 to C21. The third and fourth groups were defined according to congeners containing carbon numbers C22 to C29 and C30 to C37, respectively. The sum of the response in each group was divided by the sum of the total response of the entire mixture to estimate the percent or proportion of each group in each sample. Similarly, the influence of ring structure on sorption was examined by dividing the NAs mixture profile into seven groups based on *Z* values of 0, -2, -4, -6, -8, -10, and -12, where a *Z* value of 0 represents a structure with zero rings, -2 represents a structure with 1 ring, -4 represents a structure with 2 rings, and so on. To examine the integrated effect of carbon number and *Z* series on sorption, the NAs mixture profile was arbitrarily divided into more manageable sub-classes. The complete NAs fingerprint was divided into nine sub-classes labelled as clusters A to I (Chapter 5, Figure 5.2). The relative response measured in the NAs mixture profile was summed for each of the nine clusters. For example, the total response for cluster A was the sum of all responses detected for carbon numbers 11 to 13 and *Z* series -2 and -4. Eight out of the

nine sub-classes, each representing a cluster, were analyzed. Cluster C was not included in the analysis because no response was detected within this sub-class using ESI-MS analysis.

6.3.5 Statistical Method

All graphing and statistical analyses were conducted using Microsoft® Excel (2008 Version 12.3.0) and SigmaPlot® software (Ver 11.0 Systat Software Inc.) with a confidence level of $\alpha = 0.05$. For total NAs concentrations and water chemistry parameters, significant differences were determined using a one-way analysis of variance (ANOVA) followed by Holm-Sidak pair-wise comparisons. Data were tested for normality using the Shapiro-Wilk test and for equality of variance using the Levene median test. For the NAs compositional analyses, the arcsine of each proportional response in the congener distribution profile was taken as a variance stabilizing transformation. Transformed data were tested for normality using the Shapiro-Wilk test and for equality of variance using the Levene median test; although there were a few sample sets that did not pass these tests, the use of parametric statistics on the transformed data was continued for consistency. Statistical differences were determined using a two-way analysis of variance (ANOVA) followed by Holm-Sidak pair-wise comparisons.

6.4 Results and Discussion

6.4.1 Total Naphthenic Acids Concentrations: Input versus Control OSPW

There was no statistical difference between the total NAs concentrations ($P = 0.096$) in the Input OSPW and in the Control OSPW vessels at Days 1, 7, and 14. Similarly, there was no statistical difference ($P > 0.05$) between the Input OSPW and the OSPW in the Control vessels at Days 1, 7 and 14 for any of the general water quality parameters measured, except for pH ($P < 0.001$) (Table 6.1). Other than the slight increase in pH of the OSPW in the Control vessels, it was concluded that the experimental design and materials (not including the addition of wetland sediment for experimental manipulation) did not significantly influence the water chemistry or contribute to non-sediment related losses in total NAs concentrations during the 14-day experiment. The small increase in pH (0.2 to 0.5 pH units) was not expected to contribute to increased NAs sorption, especially since NAs are weak acids (Clemente and Fedorak, 2005). If anything, an increase in the ambient water pH should slightly increase NAs aqueous solubility and reduce their sorption on to the materials used. As a result, all data for Control OSPW at

Days 1, 7 , and 14 were pooled and thus considered as one combined representative Control sample.

Table 6.1. Total naphthenic acids concentrations and general water quality (mean ± SD) for Input OSPW compared to Control OSPW collected on Days 1, 7, and 14 (*n* values are presented in brackets).

		Time			
	Input OSPW	Control OSPW Day 1	Control OSPW Day 7	Control OSPW Day 14	Significant Difference [a] (*P* value)
Naphthenic Acids (mg/L)	62 ± 4.6 (3)	60 ± 0.0 (3)	56 ± 7.0 (3)	67 ± 2.1 (3)	No (*P* =0.096)
pH	7.8 ± 0.0 (4)	8.2 ± 0.0 (3)	8.3 ± 0.0 (3)	8.0 ± 0.0 (3)	Yes (*P* <0.001)
Conductivity (μS/cm)	2731 ± 12 (4)	2738 ± 4 (3)	2740 ± 11 (3)	2736 ± 12 (3)	No (*P* =0.697)
Alkalinity (mg/L as $CaCO_3$)	868 ± 17 (4)	837 ± 6 (3)	855 ± 22 (3)	850 ± 14 (3)	No (*P* =0.165)
Hardness (mg/L as $CaCO_3$)	71 ± 1 (4)	73 ± 1 (3)	71 ± 3 (3)	69 ± 1 (3)	No (*P* =0.186)

[a]Statisitcal differences were determined using analysis of variance (ANOVA) followed by Holm-Sidak pairwise comparisons.
OSPW = oil sands process-affected waters.

6.4.2 Naphthenic Acids Sorption

The results of the batch-reactor experiment demonstrated a rapid (1 day), decrease in total NAs concentrations in the test OSPW vessels (e.g., Day 1, Day 7, and Day 14) containing the wetland sediments compared to the Input and Control OSPW without wetland sediments (P <0.001) (Figure 6.3). No further decrease in total NAs concentration was observed between Day 1 and Day 14 (P >0.05) in the Test OSPW vessels, suggesting that the available binding sites on the sediment were fully saturated after 1 day of agitation. The mean percent reduction in total NAs concentrations in the water of the test batch-reactor vessels from Day 1 to 14 was approximately 67% relative to the mean NAs concentrations in the Control OSPW during the 14-day test period.

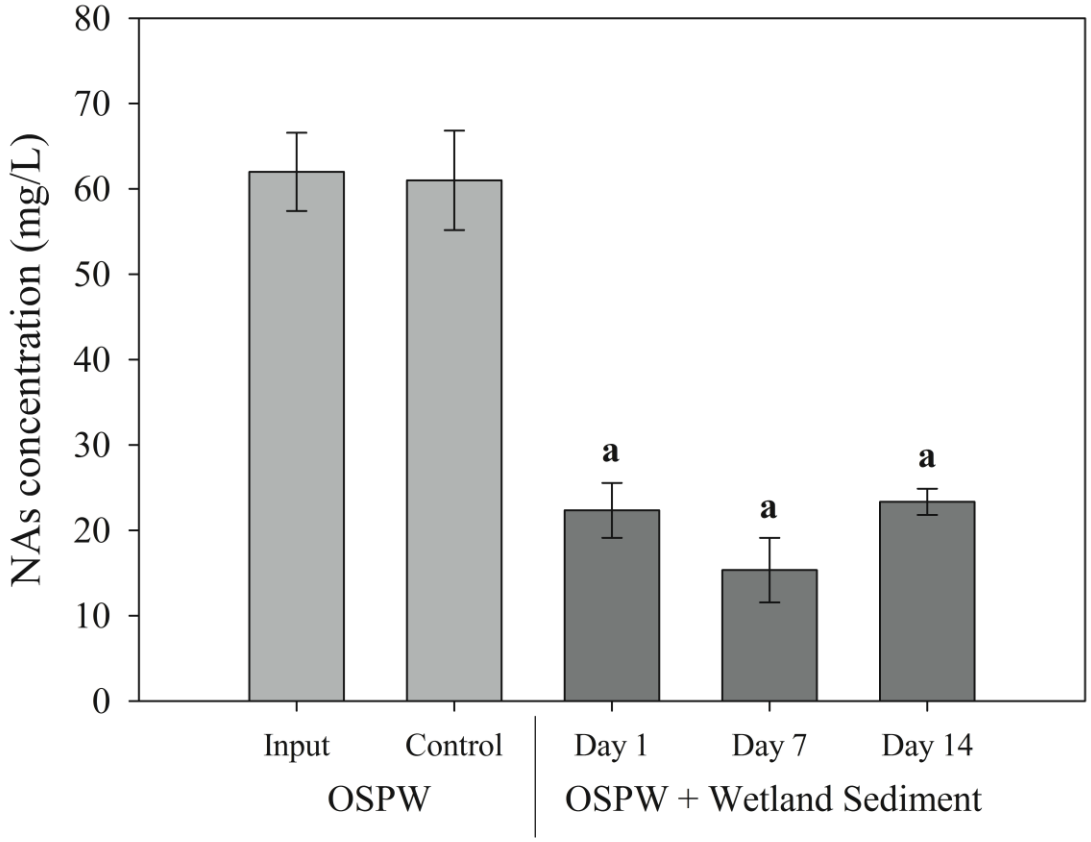


Figure 6.3. Total naphthenic acids (NAs) concentrations in Input (n =3) and Control (n =9) OSPW, and in the test OSPW vessels containing wetland sediments at Days 1, 7, and 14 (n =3 at each time point). [a]Denotes a significant difference from Input and Control OSPW as determined by analysis of variance and Holm-Sidak pairwise comparisons (P <0.001). No statistical differences in total NAs concentrations were observed among test vessels at Days 1, 7 and 14.

This extensive and rapid decrease was not completely unexpected, although this level of sorption is not typically observed for NAs in detention ponds and settling basins in the field. The mean pH of OSPW in the test vessels containing wetland sediments on Days 1, 7, and 14 was 7.0 ± 0.1 (mean ± SE), which was approximately 1.0 pH unit lower than the pH of the Input and Control OSPW (Table 6.2). It was possible that the pH depression in the test vessels increased the proportion of un-ionized/ionized NAs thus promoting their sorption to the sediments. However, this small difference was unlikely to cause such a large effect and probably only slightly enhanced the sorptive effect. In the microcosm study (Chapter 4, Figures 4.3 and 4.4), total NAs concentrations in both Syncrude and Suncor OSPW were initially (at time = 0) reduced by approximately 51 to 74% and 34 to 58%, respectively, where on average the pH of OSPW was between 8.7 and 8.9 (Appendix A). This reduction was reasonably consistent with the 67% reduction observed after 1 day in this batch-reactor study, even though there was an approximate difference of 2.0 pH units between the OSPWs of the two studies.

Table 6.2. Total naphthenic acids concentrations and general water quality (mean ± SD) for Input and Control OSPW, and in the test vessels containing oil sands process-affected waters and sediments on Days 1, 7, and 14 (*n* values are presented in brackets).

			OSPW Mixed with Wetland Sediment		
	Input OSPW	Control OSPW	Day 1	Day 7	Day 14
Naphthenic Acids (mg/L)	62 ± 4.6 (3)	61 ± 5.8 (9)	22 ± 3.2 (3)	15 ± 3.8 (3)	23 ± 1.5 (3)
pH	7.8 ± 0.0 (4)	8.2 ± 0.1 (9)	6.9 ± 0.0 (3)	7.1 ± 0.1 (3)	6.9 ± 0.0 (3)
Conductivity (μS/cm)	2731 ± 12 (4)	2738 ± 9 (9)	1764 ± 29 (3)	1680 ± 45 (3)	1782 ± 19 (3)
Alkalinity (mg/L as $CaCO_3$)	868 ± 17 (4)	848 ± 15 (9)	486 ± 18 (3)	497 ± 9 (3)	531 ± 3 (3)
Hardness (mg/L as $CaCO_3$)	71 ± 1 (4)	71 ± 2 (9)	117 ± 4 (3)	115 ± 9 (3)	143 ± 3 (3)

OSPW = oil sands process-affected waters.

Nevertheless, the findings presented here are consistent with the original microcosm study where the results from the FTIR analysis showed an initial decrease in NAs concentration for all treatments in both Syncrude and Suncor OSPW (Chapter 4). In the original microcosm study, it was suspected that sorption may have initially caused this decrease as test waters were introduced to the aquaria containing non-oil sands impacted wetland sediments at the beginning of the study. The slight increase in NAs concentrations observed over the next few sampling points suggested that the available binding sites on the sediment became filled and that the slow, but continuous replenishment of new OSPW (i.e., flow-through system), without agitation and re-suspension of the substrate, allowed the NAs concentrations in the water column to slowly increase. This was especially apparent in the treatments with shorter hydraulic residence times.

Although attempts were to made to minimize the disturbance of sediments during the initial introduction of OSPW into the aquaria containing the substrate, some re-suspension of fine particles from the wetland sediment was observed in all tanks. This mixing of sediment and OSPW, not unlike that of the controlled agitation in the present study, would result in a similar sorptive response of NAs. In a similar study using oil sands NAs (200 mg/L) and organic rich soils, Janfada et al. (2006) showed that the equilibrium adsorption coefficient (K_d) measured after 3 hours from test commencement was no different from that measured after 2 weeks. It was concluded that 48 hours was an adequate amount of time for NAs to reach an equilibrium sorption state, which is likely why the largest decrease in total NAs concentration occurred within 1 day and no further decreases were observed at Day 7 and Day 14 in this batch-reactor experiment.

The total organic carbon content of the wetland sediment in this batch-reactor study was 27.6 ± 0.9% (mean ± SD; n =4), and the K_d values of both model and oil sands NAs have been observed to be larger for a soil with higher organic carbon content (f_{oc} =2.7%) than for one with only a slightly lower (1.6%) organic carbon content (Peng et al., 2002; Janfada et al., 2006). In the original wetland microcosm study (Chapter 4), Syncrude OSPW NAs appeared to be more affected by initial sorption than Suncor OSPW NAs. This difference may have been related to the previous exposure of Suncor NAs to highly organic sediments in the wetland from which Suncor OSPW was collected. Conversely, Syncrude OSPW was collected from a seepage point

exiting a sand dyke. This dyke is mostly composed of mineral sand with a low organic carbon fraction. Thus, the more readily sorbed NAs might have been removed from Suncor OSPW prior to exposure in the simulated microcosms, whereas the Syncrude OSPW NAs might have retained much of this fraction. This difference may also explain why, under the short HRTs, there was a significant difference between the input NAs concentration and the final NAs concentration (after 52 weeks) for Syncrude OSPW only and not for Suncor OSPW (Chapter 4, Figures 4.3 and 4.4).

The sorption results obtained in this study should not be directly extrapolated to the aquatic reclamation environments at the AOS. The influence of the sediment or substrate type (e.g., organic carbon content) and the pH of OSPW used in reclamation landscapes would affect the partitioning behaviour of NAs, but likely not to the extent observed here. Sorption experiments conducted using agitation or mixing are known to over estimate partitioning coefficients due to increases in binding surface area and the decrease in boundary layer thickness around adsorbent particles (Al-Qodah, 2000). The more mineral-rich substrates found in deeper aquatic environments in the field are unlikely to undergo physical disturbance via wind or wave action. The partitioning of NAs will be limited to the upper few millimetres or centimetres of the soil/water interface where the degree of saturation of the available binding sites will be influenced by the organic carbon content (f_{oc}), pH, and by the presence of indigenous microorganisms adapted to degrade NAs in these environments. However, in shallower wetlands with highly organic substrates, that are comparatively more prone to both physical and biological turbation, the sorptive capacity of the substrate may be much higher.

6.4.3 Preferential Sorption of Naphthenic Acids Congeners

To determine if there was selective sorption of NAs onto the highly organic wetland substrate, potential changes in the fractional abundance of NAs congeners based on carbon number, *Z* series, and arbitrarily defined clusters were examined in the batch-reactor vessels over time. Both the carbon content of the substrate and the molecular structure of the sorbate can affect the partitioning coefficient (Fetter, 2001). On average, the fractional distribution of NAs congeners in each carbon number group remained largely unchanged among the four water types (Input, Control, Day 1, and Day 7) despite the significant overall sorption observed (Figure 6.4 and

Table 6.3). The greatest changes occurred in the C14 to C21 carbon number group where there were reductions in abundance by 9 and 13% in the test OSPW on Day 1 relative to the Control and Input OSPW, respectively. This result was not statistically significant, but it was in agreement with a previous study conducted by Janfada et al. (2006) where it was determined that oil sands NAs with carbon numbers in the range of 13 to 17, for all *Z* series groups, appeared to sorb preferentially.

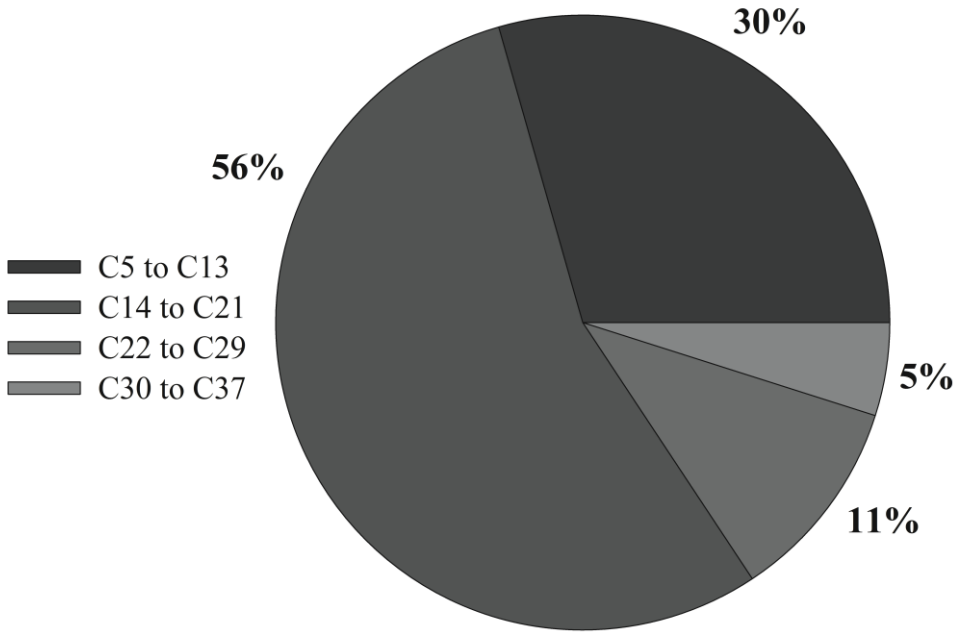


Figure 6.4. Fractional abundance of naphthenic acids (NAs) congeners in select carbon number groups averaged among the four water types (Input, Control, Day 1, and Day 7; *n* =4 for each water type) as determined by electrospray ionization mass-spectrometry (ESI-MS).

Table 6.3. Fractional abundance (mean ± SD) of naphthenic acids congeners, based on select carbon number groups, in test OSPW on Day 1 and Day 7 relative to Input and Control OSPW (*n* values are presented in brackets).

Carbon Number Group	Input OSPW	Control OSPW	OSPW Mixed with Wetland Sediment	
			Day 1	Day 7
C5 to C13	27 ± 1.5 (22)	30 ± 1.7 (22)	30 ± 1.2 (22)	31 ± 1.2 (22)
C14 to C21	63 ± 1.0 (50)	59 ± 1.0 (50)	50 ± 0.6 (50)	50 ± 0.6 (50)
C22 to C29	8 ± 0.1 (56)	8 ± 0.1 (56)	13 ± 0.1 (56)	13 ± 0.1 (56)
C30 to C37	2 ± 0.0 (56) [a]	3 ± 0.0 (56)	7 ± 0.0 (56) [a]	6 ± 0.1 (56)

[a]Denotes a significant difference (P =0.007) over time between Input OSPW and test OSPW on Day 1 within the C30 to C37 group. Statistical differences were determined using analysis of variance (ANOVA) and Holm-Sidak pairwise comparisons.
OSPW = oil sands process-affected waters.

Statistical analysis did reveal a significant interaction between the factor of time and carbon number group (P <0.001), where a change in the proportion of the carbon number group was dependent on time (i.e., sorption). Post-hoc comparisons revealed that the only interaction was due to a 5% increase in the abundance of the C30 to C37 group in the Input sample (2%) relative to the test OSPW on Day 1 (7%) (P =0.007). However, the ESI-MS analysis provides only relative and not absolute abundances, so this could be a result of an apparent "shift" in the abundances. The reduction in total NAs concentration should be associated with a decrease in the absolute abundance of a given carbon number group if there was preferential sorption. However, since the estimation of abundances are relative, a decrease in the abundance in one group of congeners could result in the increase in the relative abundance of another, not unlike that observed in the C30 to C37 group in this study (Table 6.3).

Examination of the distribution of NAs congeners based on Z series (i.e., degree of cyclization) did not reveal any significant differences in the test OSPW on Days 1 and 7 relative to the Input and Control OSPW due to sorption (Table 6.4). Similar to the results based on carbon number groups, there was no significant interaction between time (i.e., sorption) and Z series group (P =1.00). The average fractional distribution of NAs congeners of each Z series from Z = 0 to -12, remained relatively unchanged among the four water types (Figure 6.5 and Table 6.4). The

majority of the NAs congeners fell within the Z = -4 group (25 to 30%). There are a limited number of studies that have looked at the influence of sorption on the distribution of Z series in NAs mixtures directly. Peng et al. (2002) indicated that there would be little adsorption of model, single-ring naphthenic acids by soil, however, using oil sands NAs, Janfada et al. (2006) indicated that compounds in the Z = -2 NAs family appeared to sorb preferentially. In contrast, the results presented here show that the largest preferential sorption occurred for the Z = -4 series. Even though it was not statistically significant, the relative abundance of the Z = -4 series decreased by 6% in the test OSPW on Day 7 relative to the Control OSPW. There was also a slight corresponding increase in the fractional abundance of the Z = -2 series.

Table 6.4. Fractional abundance (mean ± SD) of naphthenic acids congeners, based on Z series groups, in test OSPW on Day 1 and Day 7 relative to Input and Control OSPW (n values are presented in brackets).

Z Series Group	Input OSPW	Control OSPW	OSPW Mixed with Wetland Sediment	
			Day 1	Day 7
0	19 ± 0.4 (32)	13 ± 0.4 (32)	17 ± 0.4 (32)	18 ± 0.6 (32)
-2	19 ± 0.8 (31)	19 ± 0.7 (31)	21 ± 0.7 (31)	20 ± 0.6 (31)
-4	30 ± 1.7 (28)	31 ± 1.9 (28)	26 ± 1.3 (28)	25 ± 1.2 (28)
-6	19 ± 1.2 (26)	18 ± 1.1 (26)	15 ± 0.8 (26)	16 ± 0.8 (26)
-8	7 ± 0.4 (24)	7 ± 0.3 (24)	8 ± 0.2 (24)	8 ± 0.3 (24)
-10	6 ± 0.3 (22)	6 ± 0.3 (22)	7 ± 0.3 (22)	6 ± 0.2 (22)
-12	6 ± 0.4 (20)	6 ± 0.4 (20)	7 ± 0.3 (20)	7 ± 0.4 (20)

OSPW = oil sands process-affected waters.

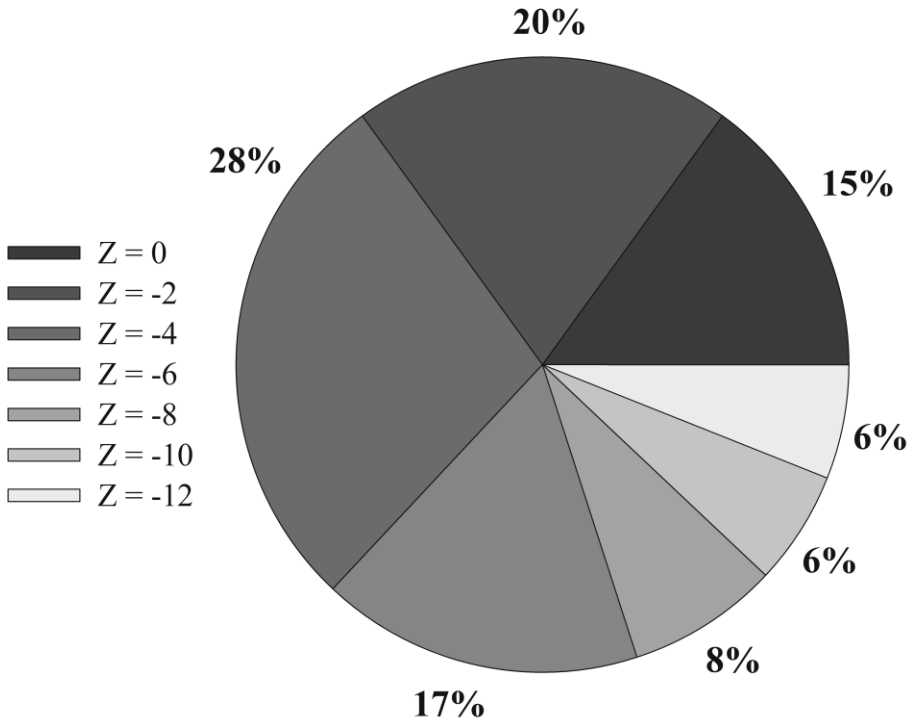


Figure 6.5. Fractional abundance of naphthenic acids (NAs) congeners in each *Z* series group averaged among the four water types (Input, Control, Day 1, and Day 7; *n* =4 for each water type) as determined by electrospray ionization mass-spectrometry (ESI-MS).

Further assessment of changes in the fractional distribution of NAs congeners based on structural clustering (clusters A to I) did not reveal any significant differences in the test OSPW on Days 1 and 7 relative to the Input and Control OSPW due to sorption (*P* =0.991) (Table 6.5). The average fractional distribution of NAs congeners in clusters A to I remained unchanged among the four water types (Input, Control, Day 1, and Day 7) despite the significant overall sorption observed (Figure 6.6 and Table 6.5). Gervais (2004), also found no changes in the three dimensional signature of NAs in groundwater after a 15% loss in total mass attributed to sorption. It was suspected that the lack of an observed change in the NAs distribution was a result of the type of NAs used (low in high molecular weight compounds) and the minimal reduction in overall NAs concentration. However, in this study, clusters G, H and I are associated with the largest, most complex NAs congeners with the highest molecular weights. The NAs in these three clusters have carbon numbers ranging from 17 to 20 and can contain up

to 6 rings (Chapter 5, Figure 5.2). Clusters G, H, and I made up approximately 29% (Table 6.5) of the total proportion of NAs in the mixture and, combined with the fact that there was a 67% reduction in total NAs concentration (Figure 6.3), it was surprising that no changes in the distribution of NAs congeners within these clusters were observed.

Table 6.5. Fractional abundance (mean ± SD) of naphthenic acids congeners, based on arbitrarily defined clusters labelled A to I, in test OSPW on Day 1 and Day 7 relative to Input and Control OSPW (*n* values are presented in brackets).

Cluster	Input OSPW	Control OSPW	OSPW Mixed with Wetland Sediment	
			Day 1	Day 7
A	23 ± 2.7 (6)	26 ± 2.9 (6)	28 ± 2.2 (6)	26 ± 2.1 (6)
B	6 ± 1.3 (2)	6 ± 1.1 (2)	8 ± 1.2 (2)	8 ± 1.5 (2)
C [a]	–	–	–	–
D	24 ± 1.9 (6)	23 ± 2.2 (6)	21 ± 1.0 (6)	20 ± 1.1 (6)
E	17 ± 1.9 (6)	16 ± 1.9 (6)	12 ± 1.3 (6)	14 ± 1.3 (6)
F [b]	1 (1)	1 (1)	1 (1)	1 (1)
G	12 ± 0.6 (8)	10 ± 0.4 (8)	13 ± 0.5 (8)	14 ± 0.6 (8)
H	8 ± 0.2 (8)	7 ± 0.3 (8)	8 ± 0.3 (8)	8 ± 0.2 (8)
I	9 ± 0.3 (7)	9 ± 0.4 (7)	9 ± 0.3 (7)	9 ± 0.5 (7)

[a]No NAs congeners were detected in cluster C for any of the water types using electrospray ionization mass spectrometery (ESI-MS).
[b]Means ± SDs could not be determined because only one value was reported for cluster F in all water types.
OSPW = oil sands process-affected waters.

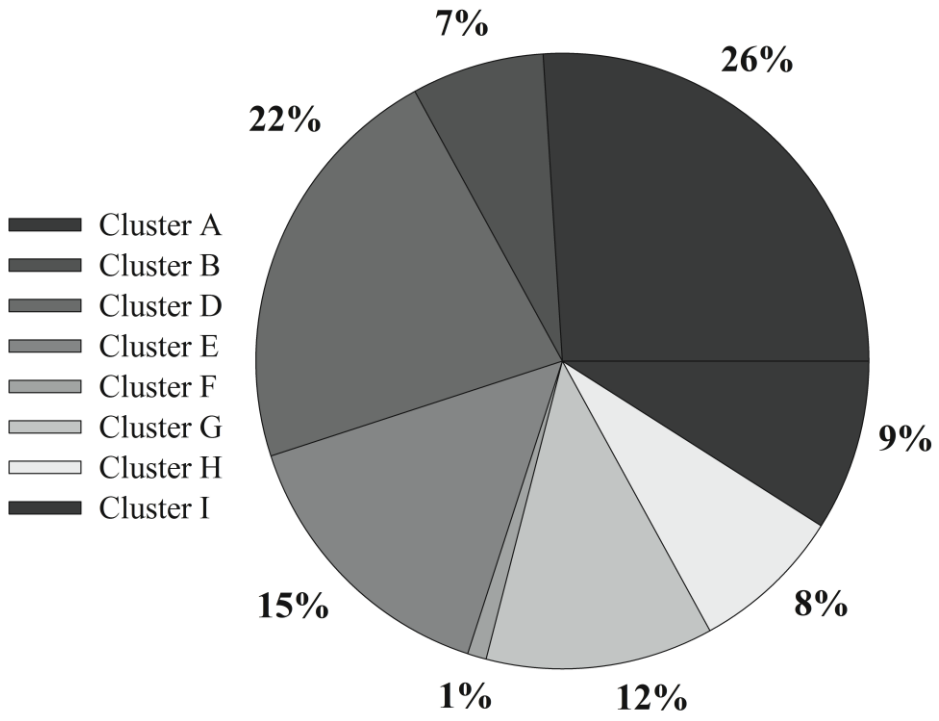


Figure 6.6. Fractional abundance of naphthenic acids (NAs) congeners in each arbitrarily defined cluster (A to I) averaged among the four water types (Input, Control, Day 1, and Day 7; *n* =4 for each water type). Cluster C was not included in the analysis because no response was detected within this sub-class using electrospray ionization mass spectrometry (ESI-MS).

Naphthenic acids are amphiphilic molecules containing a hydrophilic carboxylic acid moiety and a hydrophobic alkyl group (i.e., surfactant properties). Within a given NAs mixture, the more complex, heavier molecular weight NAs (i.e., bulkier hydrocarbon chain), such as those present in clusters G, H, and I, should in theory be more hydrophobic. Hydrophobicity is a driving force for weak physical adsorption processes where a sorbate will partition from the aqueous phase to a sorbent (e.g., soil or sediment surface) (Fetter, 2001; Peng et al., 2002). Adsorption mechanisms associated with van der Waals – London, ion-dipole, and ion-induced dipole forces (Peng et al., 2002), should have dominated the sorption process and resulted in the fractional abundance of the NAs in clusters G, H, and I to decrease. However, results from this sorption study suggest that there was no difference between the hydrophobicity of the heavier molecular weight acids (clusters G, H, and I) and that of the lower molecular weight acids (clusters A, B,

D, and E), and/or that the ESI-MS analysis was not sensitive or selective enough to detect potentially subtle differences in sorption between the NAs congener groups.

It is possible that the ESI-MS analysis, which separates and identifies NAs based on carbon number and *Z* series only, does not have the resolution to identify other structural characteristics of NAs that can alter their hydrophobicity or contribute to stronger sorption interactions. For example, it has been shown that increased alkyl substitution on NAs results in a decrease in hydrophobicity (Han et al., 2008; Martin et al., 2008). Moreover, high molecular weight NAs have been shown to be associated with increased carboxylic acid content (i.e., additional carboxylic acid functional groups), which would decrease hydrophobicity (Frank et al., 2009). Frank et al. (2009) have surmised that larger molecular weight NAs containing multiple carboxylic acid moieties would be less hydrophobic than lower molecular weight NAs based on their observed toxicological differences. Individually or in combination, increased alkyl substitution and carboxylic acid content could reduce the hydrophobicity and increase the polarity of the heavier molecular weight NAs resulting in reduced hydrophobic interactions with sediments. In the same way, an increase in the polar interactions based on hydrogen bonding, or other $\pi - \pi$ electron interactions (Fischer, 2002), would result in the reduction of total NAs concentrations without preferential selectivity based on hydrophobicity. Perhaps differences in the fractional abundances of NAs congeners associated with sorption could be detected using analyses that include information with respect to alkyl substitution (Bataineh et al., 2006) and carboxylic acid content (Frank et al., 2009).

6.5 Conclusions

The quantification of NAs sorption to wetlands sediment provided an enhanced understanding of the observed reduction in total NAs concentrations observed in the previous wetland microcosm experiment (Chapter 4). The results from the sorption study demonstrate that sorption can have a significant influence on the reduction of NAs and that the explanation for the initial decrease in the previous microcosm experiment was likely the result of sorption. However, the influence of sorption on the overall dissipation of NAs in the microcosms during the 52-week period was expected to be minimal due to the continuous input of OSPW containing NAs and the rapid saturation of available binding sites in the wetland substrate. The lack of evidence for

preferential sorption based on carbon number, *Z* series, and structure-based clusters observed in the present sorption experiment, in addition to the structure-persistence relationship (signature of biodegradation) observed in the wetland microcosm study, further supports the argument that the reduction in total NAs concentrations in the previous wetland microcosm study was greatly influenced by biodegradation. In treatments with long HRTs, the sorption effect was likely overshadowed by the increased rate of biodegradation. Had sorption been the dominant long-term process in these treatments, the structure-persistence relationship of NAs in the water column would not have been observed. Furthermore, the potential sorption of OSPW NAs to substrates with high organic carbon content in designed wetlands may enhance the mitigative capabilities of these reclamation landscapes at the AOS. Investigations into understanding sorption kinetics without substrate agitation, and concurrent toxicological evaluations are warranted, as is a better understanding of the actual structures and physiochemical properties of NAs in OSPW.

7.0 GENERAL DISCUSSION

7.1 Synopsis

The Athabasca Oil Sands (AOS) located in northeastern Alberta, Canada, has recently befallen high levels of public scrutiny and significant negative attention both in Canada and abroad (e.g., USA and Europe) due to their overall environmental footprint and implications associated with large inventories of liquid tailings and oil sands process-affected waters (OSPW). These waters are generated and stored on-site by commercial operators involved in the surface mining of oil sands. The aqueous extraction of bitumen from oil sands ore, and its upgrading to light sweet oil for export, requires approximately 2 to 2.5 m^3 of fresh water to produce 1 m^3 of synthetic crude oil. As production rates increase with the expansion of commercial activity, so does the production of liquid tailings and OSPW. It is estimated that greater than 1 billion m^3 of OSPW are currently stored on-site in various settling and holding ponds, but with the understanding that this water must eventually be reclaimed.

The reclamation of OSPW has become a significant priority for all stakeholders due to increasing concerns over environmental impacts of long-term storage and the potential seepage from holding ponds into the natural environment. Freshly produced OSPW is toxic to aquatic organisms and the toxicity has largely been linked to a relatively persistent group of dissolved organic acids known as naphthenic acids (NAs). Constructed or opportunistic lakes and wetlands offer a strategy for the bioremediation and reclamation of OSPW at the AOS. It is believed that successful reclamation of OSPW will require a significant reduction in total NAs concentrations and the removal of the toxic character of OSPW. Due to the lack of water quality guidelines for NAs in Canada, performance-based assessments using toxicological evaluations are needed for monitoring potential detrimental effects.

A potential role of wetlands in oil sands reclamation is to facilitate and stimulate NAs biodegradation, and to reduce the overall toxicity of OSPW to aquatic organisms. The natural degradation of NAs in OSPW is slow and incomplete, however, evidence based on the distribution and fractional abundance of NAs congeners suggests that certain components of the NAs mixture may degrade selectively, which in turn may be correlated with a decrease in aquatic toxicity. Accordingly, the general objectives of this research were to determine if degradation of

NAs in wetland environments could be enhanced by manipulating various physical and chemical factors, to describe and quantify the selective degradation or dissipation of NAs congeners, and to correlate observed changes in total NAs concentration and composition to changes in the aquatic toxicity of OSPW.

A preliminary toxicological assessments of OSPW in this research suggested that salinity had a greater influence on OSPW toxicity to some freshwater organisms than previously expected. Although the majority of the aquatic toxicity has been linked to the presence of NAs, the presence of dissolved salts can have significant impacts on some freshwater invertebrates, such as *Ceriodaphnia dubia.* Moreover, a mixture toxicity assessment using *C. dubia* showed that salts present in combination with NAs in OSPW would result in a less than additive or antagonistic effect, which was consistent with the findings from a separate study conducted using fish (Nero et al., 2006). Due to its tolerance for dissolved salts, the Microtox® system proved to be better suited for tracking NAs toxicity than *C. dubia* and was therefore used as the primary toxicity bioassay in the simulated wetland microcosm study.

Using a flow-through, simulated wetland laboratory microcosm experiment, it was demonstrated that the reduction in total NAs concentration was dependent upon hydraulic retention time (HRT), but appeared to be unaffected by nutrient addition (nitrogen and phosphorus). Relative to treatments with a short HRT, treatments with a long HRT were more effective in reducing total NAs concentrations. However, even under optimized laboratory conditions over a period of one year, the complete removal of NAs did not occur. Furthermore, the complete removal of NAs was not required to eliminate the initial acute toxicity of OSPW maintained under both long and short HRTs. Conversely, as the total NAs concentrations slowly dissipated in the OSPW treatments (even those with the long HRT), the chronic aquatic toxicity (Microtox® EC_{20}s) remained relatively unchanged.

Examination of the NAs fingerprint profiles revealed a structure-persistence relationship consistent with selective biodegradation. Naphthenic acids congeners with higher carbon numbers and higher degrees of cyclization (i.e., a greater number of rings) were the most persistent. The NAs fingerprint profile was also divided into nine arbitrarily defined clusters,

labelled A to I, to identify the most persistent groups of congeners within a complex OSPW NAs mixture. This approach identified the two most persistent fractions of NAs homologues as clusters H and I (representing NAs congeners with carbon numbers 17 to 20 and 3 to 6 rings), the persistence of which may explain the lack of correlation between the predominantly unchanged toxicological response (EC_{20}) as measured by Microtox® and the observed reduction in total NAs concentration.

The observed structure-persistence relationship, as a signature of biodegradation, confirmed that the degradation of NAs in simulated wetland microcosms were the result of biological processes. A subsequent sorption study using similar materials to those used in the original microcosm study highlighted the potential for abiotic processes (e.g., sorption) to have contributed to the observed reduction in total NAs concentrations. Interestingly, no selectivity or preferential sorption of specific NAs congeners was observed. Based on the structure-persistence relationship observed in the microcosm study, the continuous replenishment of OSPW and NAs in the microcosms, and the lack of selectivity observed in the separate sorption experiment, it was concluded that biotic processes dominated the overall reduction in total NAs concentrations over the course of the 1-year microcosm experiment.

It is expected that the results of this research will contribute to a greater understanding of the design and criteria required to optimize wetlands and other aquatic environments for NAs remediation and OSPW detoxification at the AOS. The complete removal of NAs may not be necessary to meet performance-based criteria, as reductions or changes, via biological processes, in specific groups of NAs congeners may be sufficient to mitigate the associated acute toxicity of OSPW to fish. Aquatic systems with long HRTs, highly organic substrates, and increased dissolved oxygen concentrations provide environments better suited for the reclamation of OSPW, but still leave behind a persistent fraction of NAs (including classical and non-classical NAs), which is associated with the chronic aquatic toxicity of OSPW. Natural bioremediation in lakes or wetlands could play a critical role in meeting inevitable regulatory requirements and obtaining approvals for lease closure landscapes, however as a stand-alone reclamation strategy, its potential may be limited.

7.2 Challenges

The extraordinary complexity of NAs and OSPW present a number of challenges for both chemical and biological investigations. At the same time, these challenges have also contributed to innovative advancement in analytical technology and enhanced the current understanding of NAs within a relatively short period of time. Two main challenges that were encountered during completion of this research study were the issue of salinity in OSPW, and the high variability in NAs analysis. These two issues must be addressed before more advanced research investigations into NAs biodegradation and their associated toxicity is conducted, and before recommendations for the development of regulatory guidelines in the context of reclamation at the AOS can be made with confidence.

7.2.1 Salinity

Although the aquatic toxicity of OSPW has largely been attributed to the presence of NAs, the influence of dissolved salts on the toxicity of NAs to freshwater organisms is generally unclear. Dissolved organic compounds can potentially be reduced by bioremediation, but the removal of high levels of salinity would likely involve some level of physical treatment potentially requiring long-term management and investment by oil sands operators. The present research demonstrated that salinity and other compounds within the complex OSPW mixture can influence the overall toxicity to aquatic organisms, such as *C. dubia*. In addition, high levels of salts can also interfere with the chemical analysis of NAs using mass spectrometry.

Previous toxicological assessments of OSPW with daphnids have not highlighted the interaction between salts and NAs. Experimental reclamation ponds at the AOS containing highly saline OSPW have been observed to support *C. dubia* and other aquatic organisms. In contrast, laboratory daphnids appear to be more sensitive to the total salt content than those found in the field. These field organisms may have built up some level of tolerance to salinity (whether physiological or genetic) over generations of exposure to OSPW contained within detention ponds. After exposure to OSPW, the laboratory cultured *C. dubia* may have experienced some initial salt shock resulting in their decreased ability to maintain ion homeostasis. It is plausible that the observed lethal and sublethal effects were further exacerbated by the presence of NAs, which themselves can also lead to the disruption of ion homeostasis via general narcosis (Frank

et al., 2008; Frank et al., 2010). Future investigations should try and identify the influence of the individual salt constituents present in OSPW on the combined toxicological effects of NAs. It would also be interesting to try and raise salt tolerant *C. dubia* in the laboraotry, or use *C. dubia* that have been isolated from saline environments in the field, in bioassays using OSPW. Assuming no pre-exposure to NAs as well, these organisms may be better suited to track NAs toxicity as a result of their increased tolerance to dissolved salts, and provide information on the adaptation of organisms already inhabiting or potentially inhabiting reclamation landscapes, pre- and post-resource development.

The co-occurrence of salts and NAs can also hamper the mass spectrometric analysis of NAs. For example, the salts in OSPW or those associated with extracted NAs, can cause ion suppression during analysis. Ion suppression matrix effects have been shown to drastically reduce apparent NAs concentrations and complicate the mass spectra (Appendix C). One way to avoid ion suppression is to use a preparatory clean-up procedure before analysis. This can be accomplished using a number of techniques, including commonly used solid phase extraction (SPE) ENV+ cartridges. The degree of ion suppression and its associated effects are difficult to estimate *a priori* and are generally unknown until samples are analyzed with and without taking ion suppression into consideration. In the mass spectra produced by ESI-MS analysis, ion suppression or detector saturation due to the presence of salts, can lead to poor chromatographic peak shape (e.g., flattened and bimodal as opposed to unimodal peak typical of OSPW NAs), and obscure the detection of high mass non-NAs peaks, which if un-accounted for can lead to erroneously high total NAs concentration measurements.

7.2.2 Naphthenic Acids Analytical Variability

Perhaps the single most important challenge encountered in this study was the variability associated with NAs analysis. The unusually high variability recorded was due to a combination of factors including, but not limited to, the analytical techniques used, the structural complexity and chemistry of NAs, and the source and storage of OSPW and NAs.

7.2.2.1 Analytical Techniques

Four different analytical techniques were used in this research for the quantification and characterization of NAs with varying degrees of success. These techniques ranged from spectroscopic, to low- and high-resolution mass spectrometry using different separation techniques. Contrary to published literature (Clemente, 2004; Clemente and Fedorak, 2005), it was determined early in the research program that the GC/MS method did not have the required resolution to detect subtle differences in the composition of NAs based on carbon number and *Z* series in complex samples from the wetland microcosm study. The HPLC/QTOC-MS and ESI-MS methods proved to be more useful, but they did not always provide consistent results. In addition, the industry standard for the quantification of total NAs at the commencement of this research program was the FTIR method. This non-specific analysis, based on the spectroscopic identification of carboxylic acid functional groups, is subject to high interferences from naturally present dissolved organic carbon (DOC) and carboxylic acid functional groups not associated with NAs. This is especially problematic in field waters from productive wetlands with high levels of organic decomposition (and thus DOC), and might be the cause of the high variability in the results from the FTIR analysis observed in this study. Regardless of the reason, such differences question the accuracy of reported NAs concentrations and suggest caution be used when interpreting absolute NAs concentrations reported here and elsewhere in the literature.

The variability for quantifying total NAs concentrations among the different NAs analytical techniques can be as high as 20 to 25%. For example, the FTIR method routinely over estimates total NAs concentrations in environmental samples by 11% from those measured using the HPLC method (Clemente and Fedorak, 2005). It remains undetermined which method is more accurate, but evidence suggests the latter method is more accurate due to the nature of the non-specific (i.e., interferences from the presence of naturally occurring carboxylic acids) FTIR analysis. During the analysis of total NAs concentrations in this research, replicate samples analysed using ESI-MS by the same laboratory showed variability as high as 76% (data not shown). Replicate samples analyzed using different methods (FTIR and ESI-MS) by different laboratories differed by as much as 50 to 88% (data not shown). The large discrepancies in total NAs concentrations reported were suspected to arise from incomplete ionization of the sample, and ion suppression due to matrix effects described previously.

7.2.2.2 Structural Complexity and Chemistry of Naphthenic Acids

The mass spectra, and fingerprints based on carbon number and *Z* series, produced by different analytical techniques also displayed different distribution patterns of NAs homologues. This is most likely related to the detection of hydroxylated intermediates, multi-carboxylated congeners, and the detection of false positives, that may or may not be excluded, depending on the type of analytical and data analysis technique used. In addition to incomplete ionization and ion suppression, these differences could also be related to differences in the internal standards that are specific to each analytical method, and to the presence of other constituents such as sulfate/sulfonate surfactants (Schramm et al., 2000), and previously unidentified elements such as nitrogen (Grewer et al., 2010) present in some isolated oil sands NAs extracts. The mass spectra of oil sands acid extracts (mostly considered to consist of classical NAs), produced using ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS), showed that <50% of the total peak abundance (for m/z ratio between 145 and 600) could be assigned to classical and oxy-NAs, and that >50% did not fit the classical definition of NAs (Grewer et al., 2010). Sulfur and nitrogen components such as the O_nS, O_nS_2, NO_n, and N_2O_n, species have all been detected in isolated oil sands NAs fractions (Headley et al., 2009; Grewer et al., 2010; Headley et al., 2011b). Furthermore, Headley et al. (2011a) illustrated, salting-out effects for the detection of acids containing O_2S and O_3S species, and enhancements in the detection of Z = -4 NAs congeners, due to the interaction between salts and oil sands acids.

All NAs analysis methods are pseudo-quantitative, and due to the lack of a consistent and common reference standard, the absolute concentrations of NAs can not be accurately determined. The HPLC/QTOF-MS analysis uses the model NA tetradecanoic acid-1-^{13}C ($C_{14}H_{28}O_2$) as an internal standard whereas, the ESI-MS analysis uses extracted OSPW NAs. Again, it is not clear which method provides the more accurate result. Using a model NA ensures more consistency between sample tests and batches of standards when they are replaced. On the other hand, its applicability to OSPW NAs is questioned as a result of the complex nature of OSPW NAs mixture and the presence of numerous compounds and NAs congeners that do not fit the classical definition of $C_nH_{2n+z}O_2$. Using an OSPW NAs extract as the standard is more appropriate, but it is more difficult to obtain and its consistency from batch to batch can not be

guaranteed. Moreover, extraction of NAs prior to analysis likely changes the NAs profile and chemical characteristics as a result of potential co-extracted species.

The extraction and isolation of a NAs extract also contributes to the overall variability in analytical results. Bulk isolation methods via acidification and subsequent liquid/liquid solvent extraction used to obtain the OSPW NAs standard for the ESI-MS analysis are highly labour intensive, requiring a significant volume of OSPW (hundreds of litres) to obtain just a few grams of NAs. For the analysis of NAs samples, solid phase extraction using an ENV+ cartridge is used in the ESI-MS analysis when required (e.g., high salinity), and a simple dilute-(with methanol) and-shoot direct injection method is used in the HPLC/QTOF-MS analysis. The NAs isolation and extraction procedures and the preparatory steps are again, additional sources of variability, and the complexity of the resulting mass spectra can be quite different depending on whether direct injection or SPE extraction and concentration was used (Appendix C). New evidence using synchrotron radiation has shown that the chemical composition of isolated NAs fractions using different extraction techniques can vary with respect to the different elements (other than carbon, hydrogen, and oxygen) that are co-isolated during the preparatory step (data not shown).

Recent studies have, in fact, shown that classical NAs make up only a fraction of the isolated organic acids that are incorrectly referred to as naphthenic acids (Grewer et al., 2010). This has coincided with a shift in the terminology used to describe the isolated NAs. Oil sands NAs are operationally defined, where the water from which they are isolated is first acidified, followed by the isolation of the organic fraction. Because this procedure is not specific for NAs defined by the general formula $C_nH_{2n+z}O_2$, the isolated fraction is now more accurately referred to as the acid-extractable organics (AEO) fraction (Grewer et al., 2010). The AEO fraction contains the classical NAs, but also contains the multi-carboxylated NAs, hydroxylated intermediates, additional natural surfactants, and other elemental constituents (e.g., nitrogen, vanadium, nickel, aluminum, boron, chromium, etc.) that are likely represented by the unassigned peaks in the mass spectra. The exact composition of the AEO varies not only with respect to the isolation and extraction procedure, but it also depends on the source of the OSPW.

7.2.2.3 Source and Storage of Oil Sands Process-Affected Waters and Naphthenic Acids

There is an inherent variability in NAs composition related to both the geological source and maturation of bitumen, even within a local formation (Chilingarian and Yen, 1978). The composition of NAs in process waters can also vary as a result of differences in the bitumen extraction procedure used by different companies (e.g., Syncrude Canada Ltd. and Suncor Energy Inc.) at the AOS. For example, the simulated wetland biodegradation study showed that Suncor OSPW NAs were less acutely toxic and more biodegradable than Syncrude OSPW NAs. It is generally accepted that ageing and maturation of process waters within retention ponds affects the concentration and composition (i.e., distribution of NAs congeners) of a given NAs mixture. Developments and site operations are in constant flux, and it is not always clear what OSPW is used, or from where it was obtained. Over time, there may be additional inputs or varying degrees of dilution due to precipitation and surface run-off, or concentration due to evaporation and the potential release of toxic constituents from an assortment of substrates used in the construction of these ponds. The collection of an OSPW sample for research from the same point at different times does not always guarantee consistency. This undefined and un-quantified variability due to the sample source is carried forward into the analytical component of research investigations, and also makes it difficult to make comparisons between studies if this information is not well documented.

The storage of OSPW and isolated NAs extracts can affect the concentration and composition of NAs making it difficult to reproduce or compare, the results from studies conducted within relatively short time frames using the same materials. It was noted during the course of this research that OSPW stored at 4°C over a period of six months resulted in differences in the concentration of NAs and its associated toxicity (data not shown). In addition, it was demonstrated that the cold storage of extracted oil sands NAs obtained from research conducted by Rogers (2003), resulted in a decrease in the total NAs concentration of the sample over time. For example, using the same FTIR method of analysis by the same laboratory, the total concentration of the extract used to prepare the NAs dilution water was 6453 mg/L. However, the concentration should have been closer to the initial measurement of 8217 mg/L taken about 3 years prior (Rogers, 2003). This suggests that either there is variability in the analysis, or that the sample had aged within the vial leading to a slight decrease in the total NAs concentration.

7.3 Recommendations for Future Research

The chemical analysis of NAs is challenging to say the least, and the reproducibility of results is a pressing issue. It is expected that the natural and analytical variability in total NAs concentrations and compositions is due to one or more of the following: differences in the bitumen ore; differences in the processing of bitumen that results in the production of OSPW that contains NAs; differences in the nature (e.g., age, substrate, and isolation from precipitation run-off and fresh OSPW input) of the retention ponds from which the OSPW and NAs are obtained; differences in the source and storage of OSPW and isolated NAs; and differences in the methods used to analyze NAs concentration and composition, including the specific extraction and isolation procedures and internal standards. The sources of natural variability and potential experimental/analytical error need to be examined systematically. It is recommended that experts in the field of NAs analysis come together to discuss, at a minimum, some consensus about the accuracy and consistency of measurements pertaining to the different analytical techniques currently used in NAs analysis. As a starting point, the goals of this truly collaborative undertaking should include: establishing a common reference standard to be used in all NAs analysis; making the analysis fully quantitative instead of pseudo-quantitative, which in turn would allow the concept of mass balance to be applied to experiments; and, conducting an inter-laboratory study with subsets of identical samples to accurately quantify the bias associated with each analytical method. Without such collaboration, the utility of NAs research remains limited. The increasing number of new technologies for NAs analysis, and the associated analytical variability and biases make it extremely difficult to compare results among different studies.

Naphthenic acids are a complex mixture dissolved in another complex mixture (OSPW). As demonstrated in this research, this situation makes it difficult to correlate changes in NAs biodegradation with the overall toxicity of OSPW, especially because of potentially confounding factors. It is recommended that toxicity studies involving isolated NAs alone, be conducted prior to whole effluent (OSPW) toxicity studies in order to determine the toxicity associated with NAs without the relative influence of the other constituents of OSPW (e.g., PAHs, ammonia, metals, salts). Additional research involving the fractionation of oil sands NAs mixtures, based on the physiochemical differences of select groups of congeners, and examining the toxicity of these

distinct fractions would contribute greatly to an understanding of the toxicity associated with select groups of NAs. Although recent studies have attempted to separate NAs mixtures and assess the toxicity associated with isolated fractions, this has met with limited success (Lo et al., 2006; Frank et al., 2008). The unequivocal demonstration of which NAs congeners are primary contributors to the NAs mixture toxicity has eluded researchers. The complete, or even partial, separation of NAs has proven to be very difficult due to the similar nature of the physiochemical properties among congeners. Recently, however, small-scale separations have lead to the identification of individual NAs from OSPW NAs mixtures (Rowland et al., 2011a; Rowland et al., 2011b). Although this information is valuable from a chemistry perspective, it does not allow for the isolation of NAs in quantities substantial enough to conduct bioassays. Perhaps, the isolation of individual NAs is not required. The fractionation of select groups of NAs congeners, having significant volumes with total concentrations that are sufficient to facilitate toxicological evaluation, would prove more valuable. Large-scale, bulk separation experiments using off-line anion exchange column chromatography were attempted as a part of this research with limited success (Appendix D). However, the use of a proprietary, co-polymer for bulk extraction and elution of NAs from OSPW provided very promising preliminary results (Appendix D) which, with further experimental refinement, could potentially result in the fractionation of NAs at adequate volumes and concentrations for toxicological assessment.

Research studies attempting to fractionate oil sands NAs mixtures are hampered by the limited resolution and high variability of current NAs analytical techniques. Although examining the fractional distribution of NAs congeners based on carbon number and *Z* series (i.e., number of rings) has provided a wealth of information, it does not provide a complete picture. As mentioned previously, changes in the distribution of NAs congeners within a given NAs mixture are relative, thus the quantification or mass balance of absolute changes in the distributions is not possible. Relative changes that are based on proportion data are sometimes unclear and difficult to interpret due to artificial increases and decreases of certain groups of NAs homologues. Furthermore, factors or structural characteristics of NAs mixtures, other than carbon number and degree of cyclization, have been shown to influence biodegradation and toxicity. This information is not part of the fingerprint analysis and includes, degree of alkyl substitution, presence of hydroxylated metabolic intermediates and multi-carboxylated NAs homologues, and

sulfur-containing co-extracted surfactants, which when analyzed concurrently with the fingerprint analysis, could enhance the understanding of the relationship among biodegradation, persistence, and toxicity.

Another characteristic of the NAs that could potentially explain their persistence and toxicity in aquatic environments is their ability to form micelles (due to their amphiphatic nature). Micelle formation is a dynamic process, and once a certain concentration of surfactant molecules dissolved in water is reached, the molecules begin to form aggregates (i.e., micelles). This threshold concentration is commonly referred to as the critical micelle concentration (CMC) and is a property of the surfactant and other factors such as temperature and electrolyte concentrations (Schramm et al., 2000). In addition, NAs that are isolated and concentrated in solvents for analysis have been known to form micelles, which could potentially lead to incomplete ionization or interferences in the spectral analysis of NAs. Thus far, no studies have investigated the potential micelle formation of NAs mixtures. Preliminary investigations performed as part of this research have shown the potential for commercial NAs to form micelles and that the CMC was lower in solutions containing higher levels of dissolved salts (Appendix E). Further research involving OSPW NAs and their ability to form micelles is recommended.

The current understanding of NAs is changing based on advanced research using both old and new analytical techniques. Oil sands NAs extracts are more complex mixtures than originally thought and also contain constituents other than the classical NAs. Previous assumptions of NAs fate and behaviour have been challenged (e.g., sorption) largely because they were based on the characteristics of commercially available NAs mixtures and model carboxylic acids. However, it is now clear that OSPW NAs behave quite differently and research may have to take a step back and re-examine their most basic physiochemical properties using newly available high resolution analytical techniques. It appears that our understanding is limited by the quality of our analytical methods and much effort has been devoted to increasing the resolution of downstream analysis, such as using advanced ionization methods and mass spectrometry instrumentation in tandem. In spite of these downstream improvements in analytical capability and resolution, it is surprising that all this new information on OSPW NAs mixtures has not been used to refine the rather crude method of isolating the AEO fraction that is associated with some of the current techniques.

Improvements or refinements made to the acid extraction part of the NAs analysis may result in less interferences from non-classical NAs, cleaner spectra, improved interpretation of results, and a greater understanding of OSPW NAs.

8.0 REFERENCES

Adams, J.J., Rostron, B.J., Mendoza, C.A., 2004. Coupled fluid flow, heat and mass transport, and erosion in the Alberta basin: implications for the origin of the Athabasca oil sands. Can. J. Earth Sci. 41, 1077-1095.

Al-Qodah, Z., 2000. Adsorption of dyes using shale oil ash. Water Res. 34, 4295-4303.

Alberta Energy and Utilities Board, 2010-2011. Alberta Ministry of Energy Annual Report.

Andersen, H.B., Buckley, J.A., 1998. Acute toxicity of ammonia to *Ceriodaphnia dubia* and a procedure to improve control survival. B. Environ. Contam. Tox. 61, 116-122.

AOSTRA, 1989. AOSTRA (Alberta Oil Sands Technology and Research Authority) technical handbook on oil sands, bitumens and heavy oils/edited by Loren G. Hepler and Chu Hsi. Technical publication number 6. AOSTRA, Edmonton, AB, Canada.

AOSTRA, 1990. AOSTRA: a 15 year portfolio of achievement. Alberta Oil Sands Technology and Research Authority, Edmonton, AB.

Armstrong, S.A., Headley, J.V., Peru, K.M., Germida, J.J., 2008. Phytotoxicity of oil sands naphthenic acids and dissipation from systems planted with emergent aquatic macrophytes. J. Environ. Sci. Health Part A Toxic/Hazard. Subst. Environ. Eng. 43, 36-42.

Arthur, J.W., West, C.W., Allen, K.N., Hedtke, S.F., 1987. Seasonal toxicity of ammonia to five fish and nine invertebrate species. B. Environ. Contam. Tox. 38, 324-331.

ASTM Standard D664-09a, 2009. Standard test method for acid number of petroleum products by potentiometric methods. ASTM International, West Conshohocken, PA, USA.

ASTM Standard D974-11, 2011. Standard test method for acid and base number by color-indicator titration. ASTM International, West Conshohocken, PA, USA.

Azur Environmental, 1989. Microtox manual: A toxicity testing handbook. 1989-1993. Azur Environmental, Carlsbad, CA, USA.

Barker, J., 1999. Mass spectrometry (2nd Ed.). John Wiley & Sons Limited, Chichester, West Sussex, UK.

Barrow, M.P., Headley, J.V., Peru, K.M., Derrick, P.J., 2004. Fourier transform ion cyclotron resonance mass spectrometry of principal components in oilsands naphthenic acids. J. Chromatogr. A 1058, 51-59.

Barrow, M.P., McDonnell, L.A., Feng, X.D., Walker, J., Derrick, P.J., 2003. Determination of the nature of naphthenic acids present in crude oils using nanospray Fourier transform ion cyclotron resonance mass spectrometry: The continued battle against corrosion. Anal. Chem. 75, 860-866.

Barrow, M.P., Witt, M., Headley, J.V., Peru, K.M., 2010. Athabasca oil sands process water: Characterization by atmospheric pressure photoionization and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Anal. Chem. 82, 3727-3735.

Barton, D.R., Wallace, R.R., 1979. The effects of an experimental spillage of oil sands tailings sludge on benthic invertebrates. Environ. Pollut. 18, 305-312.

Bataineh, M., Scott, A.C., Fedorak, P.M., Martin, J.W., 2006. Capillary HPLC/QTOF-MS for characterizing complex naphthenic acid mixtures and their microbial transformation. Anal. Chem. 78, 8354-8361.

Behar, F.H., Albrecht, P., 1984. Correlations between carboxylic acids and hydrocarbons in several crude oils. Alteration by biodegradation. Org. Geochem. 6, 597-604.

Biryukova, O.V., Fedorak, P.M., Quideau, S.A., 2007. Biodegradation of naphthenic acids by rhizosphere microorganisms. Chemosphere 67, 2058-2064.

Bishay, F.S., Nix, P.G., 1996. Constructed wetlands for treatment of oil sands wastewater: Technical Report #5. Suncor Energy Inc., Oil Sands Group. EVS Environment Consultants, North Vancouver, BC.

Brient, J.A., Wessner, P.J., Doyle, M.N., 2000. Naphthenic acids. Kirk-Othmer Encyclopedia of Chemical Technology.

Canadian Council of Ministers of the Environment, 2010. Canadian water quality guidelines for the protection of aquatic life. Ammonia. In: Canadian environmental quality guidelines. 1999, Canadian Council of Ministers of teh Environment, Winnipeg, MB, Canada.

CEATAG, 1998. Naphthenic acids background information discussion report. Alberta Department of Energy, Edmonton, AB, Canada.

Chilingarian, G.V., Yen, T.F., 1978. Introduction: Organic matter and origin of oil and tar sands. In: Chilingarian, G.V., Yen, T.F. (Eds.). Bitumens, asphalts and tar sands. Elsevier Scientific Publishing Company, Amsterdam, The Netherlands.

Clark, K.A., Pasternack, D.S., 1932. Hot water seperation of bitumen from Alberta bituminous sand. Industrial & Engineering Chemistry 24, 1410-1416.

Clemente, J.S., 2004. The characterization, analyses and biodegradation of naphthenic acids. MSc Thesis. University of Alberta, Edmonton, AB, Canada.

Clemente, J.S., Fedorak, P.M., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 60, 585-600.

Clemente, J.S., MacKinnon, M.D., Fedorak, P.M., 2004. Aerobic biodegradation of two commercial naphthenic acids preparations. Environ. Sci. Technol. 38, 1009-1016.

Clemente, J.S., Prasad, N.G.N., MacKinnon, M.D., Fedorak, P.M., 2003a. A statistical comparison of naphthenic acids characterized by gas chromatography-mass spectrometry. Chemosphere 50, 1265-1274.

Clemente, J.S., Yen, T.-W., Fedorak, P.M., 2003b. Development of a high performance liquid chromatography method to monitor the biodegradation of naphthenic acids. J. Environ. Eng. Sci. 2, 177-186.

Cowgill, U.M., Milazzo, D.P., 1991. The response of the three brood *Ceriodaphnia* test to fifteen formulations and pure compounds in common use. Arch. Environ. Contam. Toxicol. 21, 35-40.

Dai, Q., Chung, K.H., 1995. Bitumen--sand interaction in oil sand processing. Fuel 74, 1858-1864.

Davis, L.F., 1992. Case histories - The petroleum refining industry. Toxicity reduction evaluation and control. Vol. 3. Chapter 6. Technomic Publishing Co., Lancaster, Pennsylvania, USA.

Del Rio, L.F., Hadwin, A.K.M., Pinto, L.J., MacKinnon, M.D., Moore, M.M., 2006. Degradation of naphthenic acids by sediment micro-organisms. J. Appl. Microbiol. 101, 1049-1061.

Derungs, W.A., 1956. Naphthenic acid corrosion - an old enemy of the petroleum industry. Corrosion 12, 41-46.

Dokholyan, B.K., Magomedov, A.K., 1983. Effect of sodium naphthenate on survival and some physiological-biochemical parameters of some fishes. Journal of Ichthyology 23, 125-132.

Dzidic, I., Somerville, A.C., Raia, J.C., Hart, H.V., 1988. Determination of naphthenic acids in California crudes and refinery wastewaters by fluoride ion chemical ionization mass spectrometry. Anal. Chem. 60, 1318-1323.

Elshayeb, M., 2006. Determining food web impacts on experimental aquatic systems from the disposal of oil sands process-affected waste material. MSc Thesis. University of Waterloo, Waterloo, ON, Canada.

Environment Canada, 1992. Biological test method: Test of reproduction and survival using the cladoceran Ceriodaphnia dubia. EPS1/RM/21. Environment Protection Series, Conservation and Protection. Environment Canada, Ottawa, ON, Canada.

Environment Canada, 2000. Biological test method: Reference method for determining acute lethality of effluents to Rainbow Trout. EPS 1/RM/13 Second edition. Technical report. Environment Canada, Ottawa, ON, Canada.

Fan, T.P., 1991. Characterization of naphthenic acids in petroleum by fast-atom-bombardment mass-spectrometry. Energ. Fuel 5, 371-375.

Fetter, C.W., 2001. Applied hydrogeology. Prentice Hall, Upper Saddle River, N.J.

Fischer, K., 2002. Environmental analysis of aliphatic carboxylic acids by ion-exclusion chromatography. Anal. Chim. Acta. 465, 157-173.

Foght, J.M., Fedorak, P.M., Westlake, D.W.S., 1985. Microbial content and metabolic activities in the Syncrude tailings pond. Alberta Oil Sands Technology and Research Authority (AOSTRA) Journal of Research 1, 139-146.

Frank, R.A., Fischer, K., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van der Kraak, G., Solomon, K.R., 2009. Effect of carboxylic acid content on the acute toxicity of oil sands naphthenic acids. Environ. Sci. Technol. 43, 266-271.

Frank, R.A., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van Der Kraak, G., Solomon, K.R., 2008. Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. Chemosphere 72, 1309-1314.

Frank, R.A., Sanderson, H., Kavanagh, R., Burnison, B.K., Headley, J.V., Solomon, K.R., 2010. Use of a (Quantitative) Structure-Activity Relationship [(Q)Sar] Model to predict the toxicity of naphthenic acids. J. Toxicol. Env. Heal. A 73, 319-329.

FTFC, 1995a. "Volume I., Clark hot water extraction fine tailings", In: Advances in oil sands tailings research. Fine Tailings Fundamentals Consortium (FTFC), Alberta Department of Energy, Oil Sands and Research Division, Edmonton, AB.

FTFC, 1995b. "Volume II, Fine tails and process water reclamation", In: Advances in oil sands tailings research. FIne Tailings Fundamentals Consortium (FTFC), Alberta Department of Energy, Oil Sands and Research Division, Edmonton, AB.

Gabryelski, W., Froese, K.L., 2003. Characterization of Naphthenic Acids by Electrospray Ionization High-Field Asymmetric Waveform Ion Mobility Spectrometry Mass Spectrometry. Anal. Chem. 75, 4612-4623.

Gentes, M.-L., McNabb, A., Waldner, C., Smits, J., 2007a. Increased thyroid hormone levels in tree swallows *Tachycineta bicolor* on reclaimed wetlands of the Athabasca oil sands. Arch. Environ. Contam. Toxicol. 53, 287-292.

Gentes, M.-L., Waldner, C., Papp, Z., Smits, J.E.G., 2006. Effects of oil sands tailings compounds and harsh weather on mortality rates, growth and detoxification efforts in nestling tree swallows (*Tachycineta bicolor*). Environ. Pollut. 142, 24-33.

Gentes, M.L., 2006. Health assessment of tree swallows (*Tachycineta bicolor*) nesting on the Athabsaca oil sands. MSc Thesis. University of Saskachewan, Saskatoon, SK, Canada.

Gentes, M.L., Waldner, C., Papp, Z., Smits, J., 2007b. Effects of exposure to naphthenic acids in tree swallows (*Tachycineta bicolor*) on the Athabasca oil sands, Alberta, Canada. J. Toxicol. Environ. Health Part A 70, 1182-1190.

Gervais, F.M., 2004. Fate and transport of naphthenic acids in a glacial aquifer. MSc Thesis. University of Waterloo, Waterloo, ON, Canada.

Golder Associates Ltd., 2000. Framework for oil sands reclamation performace assessment: Description of landforms and terrain units. Suncor Energy Inc., Report 992-2280. Golder Associates Ltd., Calgary, AB.

Government of Alberta, 2011. Talk about oil sands. Alberta Energy. Government of Alberta, Edmonton, AB, http://www.energy.alberta.ca/OilSands/pdfs/FactSheet_OilSands.pdf (accessed July 7, 2011).

Grewer, D.M., Young, R.F., Whittal, R.M., Fedorak, P.M., 2010. Naphthenic acids and other acid-extractables in water samples from Alberta: What is being measured? Sci. Total Environ. 408, 5997-6010.

Griffiths, M., Taylor, A., Woynillowicz, D., 2006. Troubled waters, troubling trends: Technology and policy options to reduce water use in oil and oil sands development in Alberta. Report 1st Ed. The Pembina Institute, Drayton Valley, AB.

Gross, J.H., 2004. Mass spectrometry. Springer-Verlag Berlin Heidelberg, Germany.

Guevremont, R., Purves, R.W., 1999. Atmospheric pressure ion focusing in a high-field asymmetric waveform ion mobility spectrometer. Rev. Sci. Instrum. 70, 1370-1383.

Gutzeit, J., 1977. Naphthenic acid corrosion in oil refineries. Mater. Performance 16, 24-35.

Hadwin, A.K.M., Del Rio, L.F., Pinto, L.J., Painter, M., Routledge, R., Moore, M.M., 2006. Microbial communities in wetlands of the Athabasca oil sands: genetic and metabolic characterization. Fems. Microbiol. Ecol. 55, 68-78.

Han, X., MacKinnon, M.D., Martin, J.W., 2009. Estimating the in situ biodegradation of naphthenic acids in oil sands process waters by HPLC/HRMS. Chemosphere 76, 63-70.

Han, X.M., Scott, A.C., Fedorak, P.M., Bataineh, M., Martin, J.W., 2008. Influence of molecular structure on the biodegradability of naphthenic acids. Environ. Sci. Technol. 42, 1290-1295.

Hao, C.Y., Headley, J.V., Peru, K.A., Frank, R., Yang, P., Solomon, K.R., 2005. Characterization and pattern recognition of oil-sand naphthenic acids using comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry. J. Chromatogr. A 1067, 277-284.

Harms, N.J., Fairhurst, G.D., Bortolotti, G.R., Smits, J.E.G., 2010. Variation in immune function, body condition, and feather corticosterone in nestling Tree Swallows (Tachycineta bicolor) on reclaimed wetlands in the Athabasca oil sands, Alberta, Canada. Environ. Pollut. 158, 841-848.

He, Y., Wiseman, S.B., Zhang, X., Hecker, M., Jones, P.D., El-Din, M.G., Martin, J.W., Giesy, J.P., 2010. Ozonation attenuates the steroidogenic disruptive effects of sediment free oil sands process water in the H295R cell line. Chemosphere 80, 578-584.

Headley, J.V., Barrow, M.P., Peru, K.M., Derrick, P.J., 2011a. Salting-out effects on the characterization of naphthenic acids from Athabasca oil sands using electrospray ionization. J. Environ. Sci. Health Part A Toxic/Hazard. Subst. Environ. Eng. 46, 844 - 854.

Headley, J.V., Barrow, M.P., Peru, K.M., Fahlman, B., Frank, R.A., Bickerton, G., McMaster, M.E., Parrott, J., Hewitt, L.M., 2011b. Preliminary fingerprinting of Athabasca oil sands polar organics in environmental samples using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Rapid Commun. Mass Spectrom. 25, 1899-1909.

Headley, J.V., McMartin, D.W., 2004. A review of the occurrence and fate of naphthenic acids in aquatic environments. J. Environ. Sci. Health Part A Toxic/Hazard. Subst. Eviron. Eng. 39, 1989-2010.

Headley, J.V., Peru, K.M., Armstrong, S.A., Han, X.M., Martin, J.W., Mapolelo, M.M., Smith, D.F., Rogers, R.P., Marshall, A.G., 2009. Aquatic plant-derived changes in oil sands naphthenic acid signatures determined by low-, high- and ultrahigh-resolution mass spectrometry. Rapid Commun. Mass Spectrom. 23, 515-522.

Headley, J.V., Peru, K.M., Janfada, A., Fahlman, B., Gu, C., Hassan, S., 2011c. Characterization of oil sands acids in plant tissue using Orbitrap ultra-high resolution mass spectrometry with electrospray ionization. Rapid Commun. Mass Spectrom. 25, 459-462.

Headley, J.V., Peru, K.M., McMartin, D.W., Winkler, M., 2002a. Determination of dissolved naphthenic acids in natural waters by using negative-ion electrospray mass spectrometry. J. AOAC Int. 85, 182-187.

Headley, J.V., Peru, K.M., Mishra, S., Meda, V., Dalai, A.K., McMartin, D.W., Mapolelo, M.M., Rodgers, R.P., Marshall, A.G., 2010. Characterization of oil sands naphthenic acids treated with ultraviolet and microwave radiation by negative ion electrospray Fourier transform ion cyclotron resonance mass spectrometry. Rapid Commun. Mass Spectrom. 24, 3121-3126.

Headley, J.V., Tanapat, S., Putz, G., Peru, K.M., 2002b. Biodegradation kinetics of geometric isomers of model naphthenic acids in Athabasca river water. Canadian Water Resources Journal 27, 25-42.

Herman, D.C., Fedorak, P.M., Costerton, J.W., 1993. Biodegradation of cycloalkane carboxylic acids in oil sands tailings. Can. J. Microbiol. 39, 576-580.

Herman, D.C., Fedorak, P.M., MacKinnon, M.D., Costerton, J.W., 1994a. Biodegradation of naphthenic acids and the reduction of acute toxicity of oil sands tailings. In: Hinchee, R.E., Alleman, B.C., Hoeppel, R.E., Miller, R.N. (Eds.). Hydrocarbon bioremediation. CRC Press Inc., Boca Raton, FL, USA.

Herman, D.C., Fedorak, P.M., MacKinnon, M.D., Costerton, J.W., 1994b. Biodegradation of naphthenic acids by microbial populations indigenous to oil sands tailings. Can. J. Microbiol. 40, 467-477.

Hersikorn, B.D., Smits, J.E.G., 2011. Compromised metamorphosis and thyroid hormone changes in wood frogs (*Lithobates sylvaticus*) raised on reclaimed wetlands on the Athabasca oil sands. Environ. Pollut. 159, 596-601.

Holowenko, F.M., MacKinnon, M.D., Fedorak, P.M., 2000. Methanogens and sulfate-reducing bacteria in oil sands fine tailings waste. Can. J. Microbiol. 46, 927-937.

Holowenko, F.M., Mackinnon, M.D., Fedorak, P.M., 2001. Naphthenic acids and surrogate naphthenic acids in methanogenic microcosms. Water Res. 35, 2595-2606.

Holowenko, F.M., MacKinnon, M.D., Fedorak, P.M., 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Res. 36, 2843-2855.

Holroyd, P., Simieritsch, T., 2009. The waters that bind us: Transboundary implications of oil sands development. The Pembina Institute, Drayton Valley, AB, Canada.

Horne, A.J., Goldman, C.R., 1994. Limnology. 2nd edition. McGraw-Hill Inc., New York, NY, USA.

Hsu, C.S., Dechert, G.J., Robbins, W.K., Fukuda, E.K., 2000. Naphthenic acids in crude oils characterized by mass spectrometry. Energ. Fuel 14, 217-223.

Jaffe, R., Albrecht, P., Oudin, J.-L., 1988. Carboxylic acids as indicators of oil migration--I. Occurrence and geochemical significance of C-22 diastereoisomers of the (17[beta]H,21[beta]H) C30 hopanoic acid in geological samples. Org. Geochem. 13, 483-488.

Jaffe, R., Gallardo, M.T., 1993. Application of carboxylic acid biomarkers as indicators of biodegradation and migration of crude oils from the Maracaibo Basin, Western Venezuela. Org. Geochem. 20, 973-984.

Jaffe, R., Gardinali, P.R., 1990. Generation and maturation of carboxylic acids in ancient sediments from the Maracaibo Basin, Venezuela. Org. Geochem. 16, 211-218.

Janfada, A., Headley, J.V., Peru, K.M., Barbour, S.L., 2006. A laboratory evaluation of the sorption of oil sands naphthenic acids on organic rich soils. J. Environ. Sci. Heal. A 41, 985-997.

Jivraj, M.N., MacKinnon, M.D., Fung, B., 1995. Naphthenic acid extraction and quantitative analysis with FT-IR spectroscopy. Syncrude analytical manuals, 4th ed. Syncrude Canada Ltd., Edmonton, AB, Canada.

Jones, D.M., Watson, J.S., Meredith, W., Chen, M., Bennett, B., 2001. Determination of naphthenic acids in crude oils using nonaqueous ion exchange solid-phase extraction. Anal. Chem. 73, 703-707.

Kamaluddin, M., Zwiazek, J.J., 2002. Naphthenic acids inhibit root water transport, gas exchange and leaf growth in aspen (Populus tremuloides) seedlings. Tree Physiol. 22, 1265-1270.

Lai, J.W.S., Pinto, L.J., Kiehlmann, E., BendellYoung, L.I., Moore, M.M., 1996. Factors that affect the degradation of naphthenic acids in oil sands wastewater by indigenous microbial communities. Environ. Toxicol. Chem. 15, 1482-1491.

Leung, S.S., MacKinnon, M.D., Smith, R.E., 2001. Aquatic reclamation in the Athabasca, Canada, oil sands: naphthenate and salt effects on phytoplankton communities. Environ. Toxicol. Chem. 20, 1532-1543.

Leung, S.S., MacKinnon, M.D., Smith, R.E.H., 2003. The ecological effects of naphthenic acids and salts on phytoplankton from the Athabasca oil sands region. Aquat. Toxicol. 62, 11-26.

Liang, X., Zhu, X., Butler, E.C., 2011. Comparison of four advanced oxidation processes for the removal of naphthenic acids from model oil sands process water. J. Hazard. Mater. 190, 168-176.

Lister, A., Nero, V., Farwell, A., Dixon, D.G., Van der Kraak, G., 2008. Reproductive and stress hormone levels in goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Aquat. Toxicol. 87, 170-177.

Lo, C.C., Brownlee, B.G., Bunce, N.J., 2003. Electrospray-mass spectrometric analysis of reference carboxylic acids and Athabasca oil sands naphthenic acids. Anal. Chem. 75, 6394-6400.

Lo, C.C., Brownlee, B.G., Bunce, N.J., 2006. Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids. Water Res. 40, 655-664.

Mackenzie, A.S., Wolff, G.A., Maxwell, J.R., 1983. Fatty acids in biodegraded petroleums. Possible origins and significance. In: Bjoroy, M.e.a. (Ed.). Advances in organic geochemistry 1981. John Wiley & Sons Limited, Chichester, UK, pp. 637-649.

MacKinnon, M.D., 1989. Development of the tailings pond at Syncrude's oil sands plant: 1978-1987. AOSTRA (Alberta Oil Sands Technology and Research Authority) Journal of Research 5, 109-133.

MacKinnon, M.D., Boerger, H., 1986. Description of two treatment methods for detoxifying oil sands tailings pond water. Water Pollut. Res. J. Can. 21, 496-512.

MacKinnon, M.D., Gulley, J., Van Meer, T., 2001. Role of wetlands in attenuation of process-related components. CONRAD Environmental Research Group (ERG) Research Symposium, Alberta Research Council, Edmonton, AB.

MacKinnon, M.D., Kampala, G., Marsh, B., Fedorak, P.M., Guigard, S.E., 2005. Indicators for assessing transport of oil sands process-affected waters. Bringing groundwater research to the watershed scale. *Proceedings of the 4th International Groundwater Quality Conference (GQ2004)*. IAHS Publ. 297, 2005, Waterloo, ON, Canada, pp. 71-80.

Madill, R.E.A., Orzechowski, M.T., Chen, G., Brownlee, B.G., Bunce, N.J., 2001. Preliminary risk assessment of the wet landscape option for reclamation of oil sands mine tailings: Bioassays with mature fine tailings pore water. Environ. Toxicol. 16, 197-208.

Marking, L.L., 1985. Toxicity of chemical mixtures. In: Rand, G.M., Petrocelli, S.R. (Eds.). Fundamentals of aquatic toxicity: Methods and applications. Hemisphere Publishing Corporation, New York, pp. 164-176.

Martin, J.W., Barri, T., Han, X.M., Fedorak, P.M., El-Din, M.G., Perez, L., Scott, A.C., Jiang, J.T., 2010. Ozonation of oil sands process-affected water accelerates microbial bioremediation. Environ. Sci. Technol. 44, 8350-8356.

Martin, J.W., Han, X.M., Peru, K.M., Headley, J.V., 2008. Comparison of high- and low-resolution electrospray ionization mass spectrometry for the analysis of naphthenic acid mixtures in oil sands process water. Rapid Commun. Mass Spectrom. 22, 1919-1924.

McMartin, D.W., 2003. Persistence and fate of acidic hydrocarbons in aquatic environments: naphthenic acids and resin acids. PhD Thesis. Environmental Engineering. University of Saskatchewan, Saskatoon, SK, Canada.

McMartin, D.W., Headley, J.V., Friesen, D.A., Peru, K.M., Gillies, J.A., 2004. Photolysis of naphthenic acids in natural surface water. J. Environ. Sci. Heal. A 39, 1361-1383.

Meredith, W., Kelland, S.J., Jones, D.M., 2000. Influence of biodegradation on crude oil acidity and carboxylic acid composition. Org. Geochem. 31, 1059-1073.

Merlin, M., Guigard, S.E., Fedorak, P.M., 2007. Detecting naphthenic acids in waters by gas chromatography-mass spectrometry. J. Chromatogr. A 1140, 225-229.

Mishra, S., Meda, V., Dalai, A.K., McMartin, D.W., Headley, J.V., Peru, K.M., 2011. Photocatalysis of naphthenic acids in water. J. Water Resour. Prot. 2, 644-650.

Miwa, H., 2000. High-performance liquid chromatographic determination of mono-, poly- and hydroxycarboxylic acids in foods and beverages as their 2-nitrophenylhydrazides. J. Chromatogr. A 881, 365-385.

Nero, V., Farwell, A., Lee, L.E.J., Van Meer, T., MacKinnon, M.D., Dixon, D.G., 2006a. The effects of salinity on naphthenic acid toxicity to yellow perch: Gill and liver histopathology. Ecotoxicol. Environ. Safe. 65, 252-264.

Nero, V., Farwell, A., Lister, A., Van der Kraak, G., Lee, L.E., Van Meer, T., MacKinnon, M.D., Dixon, D.G., 2006b. Gill and liver histopathological changes in yellow perch (*Perca flavescens*) and goldfish (*Carassius auratus*) exposed to oil sands process-affected water. Ecotoxicol. Environ. Saf. 63, 365-377.

Nimmo, D.W.R., Link, D., Parrish, L.P., Rodriguez, G.J., Wuerthele, W., Davies, P.H., 1989. Comparison of on-site and laboratory toxicity tests: Derivation of site-specific criteria for un-ionized ammonia in a Colorado transitional stream. Environ. Toxicol. Chem. 8, 1177-1189.

Nix, P.G., Martin, R.W., 1992. Detoxification and reclamation of Suncor's oil sand tailings ponds. Environ. Toxic. Water 7, 171-188.

Partearroyo, M.A., Pilling, S.J., Jones, M.N., 1992. The effects of surfactants on the permeability of isolated perfused fish gills to urea. Comp. Biochem. Physiol. Comp. Physiol. 101, 653-659.

Patrick, R., Scheier, A., Cairns, J., 1968. The relative sensitivity of diatoms, snails, and fish to twenty common constituents of industrial wastes. The Progressive Fish-Culturist 30, 137-140.

Peng, J., Headley, J.V., Barbour, S.L., 2002. Adsorption of single-ring model naphthenic acids on soils. Can. Geotech. J. 39, 1419-1426.

Pollet, I., Bendell-Young, L.I., 2000. Amphibians as indicators of wetland quality in wetlands formed from oil sands effluent. Environ. Toxicol. Chem. 19, 2589-2597.

Purves, R.W., Guevremont, R., 1999. Electrospray ionization high-field asymmetric waveform ion mobility spectrometry - mass spectrometry. Anal. Chem. 71, 2346-2357.

Purves, R.W., Guevremont, R., Day, S., Pipich, C.W., Matyjaszczyk, M.S., 1998. Mass spectrometric characterization of a high-field asymmetric waveform ion mobility spectrometer. Rev. Sci. Intsrum. 69, 4094-4105.

Quagraine, E.K., Headley, J.V., Peterson, H.G., 2005a. Is biodegradation of bitumen a source of recalcitrant naphthenic acid mixtures in oil sands tailing pond waters? J. Environ. Sci. Heal. A 40, 671-684.

Quagraine, E.K., Peterson, H.G., Headley, J.V., 2005b. In situ bioremediation of naphthenic acids contaminated tailing pond waters in the Athabasca oil sands region-demonstrated field studies and plausible options: A review. J. Environ. Sci. Heal. A 40, 685-722.

Quesnel, D.M., Bhaskar, I.M., Gieg, L.M., Chua, G., 2011. Naphthenic acid biodegradation by the unicellular alga *Dunaliella tertiolecta*. Chemosphere 84, 504-511.

Renault, S., Lait, C., Zwiazek, J.J., MacKinnon, M., 1998. Effect of high salinity tailings waters produced from gypsum treatment of oil sands tailings on plants of the boreal forest. Environ. Pollut. 102, 177-184.

Renault, S., Paton, E., Nilsson, G., Zwiazek, J.J., MacKinnon, M.D., 1999. Responses of boreal plants to high salinity oil sands tailings water. J. Environ. Qual. 28, 1957-1962.

Rogers, V.V., 2003. Mammalian toxicity of naphthenic acids derived from the Athabasca oil sands. PhD Thesis. University of Saskatchewan, Saskatoon, SK, Canada.

Rogers, V.V., Liber, K., MacKinnon, M.D., 2002a. Isolation and characterization of naphthenic acids from Athabasca oil sands tailings pond water. Chemosphere 48, 519-527.

Rogers, V.V., Wickstrom, M., Liber, K., MacKinnon, M.D., 2002b. Acute and subchronic mammalian toxicity of naphthenic acids from oil sands tailings. Toxicol. Sci. 66, 347-355.

Rowland, S.J., Scarlett, A.G., Jones, D., West, C.E., Frank, R.A., 2011a. Diamonds in the rough: Identification of individual naphthenic acids in oil sands process water. Environ. Sci. Technol. 45, 3154-3159.

Rowland, S.J., West, C.E., Scarlett, A.G., Jones, D., Frank, R.A., 2011b. Identification of individual tetra- and pentacyclic naphthenic acids in oil sands process water by comprehensive two-dimensional gas chromatography/mass spectrometry. Rapid Commun. Mass Spectrom. 25, 1198-1204.

Roy, D., 1988. Statistical analysis of anionic detergent-induced changes in the goblet mucous cells of opercular epidermis and gill epithelium of *Rita rita* (Ham.) (Bagridae: Pisces). Ecotoxicol. Environ. Saf. 15, 260-271.

Rudzinski, W.E., Oehlers, L., Zhang, Y., 2002. Tandem mass spectrometric characterization of commercial naphthenic acids and a Maya crude oil. Energ. Fuel 16, 1178-1185.

Schramm, L.L., Smith, R.G., 1985. The influence of natural surfactants on interfacial charges in the hot-water process for recovering bitumen from the Athabasca oil sands. Colloids Surf. 14, 67-85.

Schramm, L.L., Smith, R.G., Stone, J.A., 1984. A surface-tension method for the determination of anionic surfactants in hot water processing of athabasca oil sands. Colloids Surf. 11, 247-263.

Schramm, L.L., Stasiuk, E.N., MacKinnon, M.D., 2000. Surfactants in Athabasca oil sands slurry conditioning, flotation recovery, and tailings processes. In: Schramm, L.L. (Ed.). Surfactants: Fundamentals and applications in the petroleum industry. Cambridge University Press, Cambridge, UK, pp. 365-430.

Scott, A.C., MacKinnon, M.D., Fedorak, P.M., 2005. Naphthenic acids in Athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. Environ. Sci. Technol. 39, 8388-8394.

Scott, A.C., Zubot, W., MacKinnon, M.D., Smith, D.W., Fedorak, P.M., 2008. Ozonation of oil sands process water removes naphthenic acids and toxicity. Chemosphere 71, 156-160.

Seifert, W.K., Teeter, R.M., 1969. Preparative thin-layer chromatography and high-resolution mass spectrometry of crude oil carboxylic acids. Anal. Chem. 41, 786-795.

Seifert, W.K., Teeter, R.M., Howells, W.G., Cantow, M.J.R., 1969. Analysis of crude oil carboxylic acids after conversion to their corresponding hydrocarbons. Anal. Chem. 41, 1638-1647.

Seward, J.R., Schultz, T.W., 1999. QSAR analyses of the toxicity of aliphatic carboxylic acids and salts to *Tetrahymena pyriformis*. SAR QSAR Environ. Res. 10, 557-567.

Singh, S.P.N., 1985. Synthetic Fuels - Production and Products. Vet. Hum. Toxicol. 27, 285-290.

Siwik, P.L., Van Meer, T., MacKinnon, M.D., Paszkowski, C.A., 2000. Growth of fathead minnows in oilsand-processed wastewater in laboratory and field. Environ. Toxicol. Chem. 19, 1837-1845.

Skoog, D.A., Leary, J.J., 1992. Principles of instrumental analysis (4th ed.). Harcourt Publishers, Fort Worth, TX, USA.

Smith, B.E., Lewis, C.A., Belt, S.T., Whitby, C., Rowland, S.J., 2008. Effects of Alkyl Chain Branching on the Biotransformation of Naphthenic Acids. Environ. Sci. Technol. 42, 9323-9328.

Smits, J.E., Wayland, M.E., Miller, M.J., Liber, K., Trudeau, S., 2000. Reproductive, immune, and physiological end points in tree swallows on reclaimed oil sands mine sites. Environ. Toxicol. Chem. 19, 2951-2960.

Speight, J.G., 1999. The chemistry and technology of petroleum. 3rd ed. Marcel Dekker, New York, NY.

Speight, J.G., 2000. Tar sands. Kirk-Othmer Encyclopedia of Chemical Technology.

Squires, A.J., 2005. Ecotoxicological assessment of using coke in aquatic reclamation strategies at the Alberta oil sands. MSc Thesis. University of Saskatchewan, Saskatoon, SK, Canada.

St John, W.P., Rughani, J., Green, S.A., McGinnis, G.D., 1998. Analysis and characterization of naphthenic acids by gas chromatography electron impact mass spectrometry of tert.-butyldimethylsilyl derivatives. J. Chromatogr. A 807, 241-251.

Syncrude Canada Ltd., 1995. Syncrude analytical methods. Syncrude Canada Ltd., Edmonton Research Centre, Edmonton, AB, Canada.

Takamura, K., 1982. Microscopic structure of athabasca oil sand. Can. J. Chem. Eng. 60, 538-545.

Taylor, D.G., Trudgill, P.W., 1978. Metabolism of cyclohexane carboxylic-acid by Alcaligenes Strain W1. J. Bacteriol. 134, 401-411.

Tissot, B.P., Welte, D.H., 1984. Petroleum formation and occurrence. Springer-Verlag, New York, NY, USA.

Tomczyk, N.A., Winans, R.E., Shinn, J.H., Robinson, R.C., 2001. On the nature and origin of acidic species in petroleum. 1. Detailed acid type distribution in a California crude oil. Energ. Fuel 15, 1498-1504.

Turnbull, A., Slavcheva, E., Shone, B., 1998. Factors controlling naphthenic acid corrosion. Corrosion 54, 922-930.

U.S. Environmental Protection Agency, 1990. Trimmed Spearman-Karber (TSK) program, Ver. 1.5. Ecological Monitoring Research Division, Cincinnati, OH.

U.S. Environmental Protection Agency, 1993. Linear interpolation method for sub-lethal toxicity: The Inhibiton Concentration (ICp) approach, Ver. 2.0. Environmental Research Laboratory, Duluth, MN.

U.S. Environmental Protection Agency, 2009. Draft 2009 update aquatic life ambient water quality criteria for ammonia - freshwater. EPA-822-D-09-001. U.S. Environmental Protection Agency, Office of Water, Washington, DC, USA.

Uzhdavini, E.R., Glukharev Iu, A., 1984. Toxic properties of synthetic naphthenic and alkylbenzoic acids]. Gig. Tr. Prof. Zabol. 48-49.

van den Heuvel, M.R., Power, M., MacKinnon, M.D., Dixon, D.G., 1999. Effects of oil sands related aqautic reclamation on yellow perch (*Perca flavescens*). II. Chemical and biochemical indicators of exposure to oil sands related waters. Can. J. Fish. Aquat. Sci. 56, 1226-1233.

van den Heuvel, M.R., Power, M., Richards, J., MacKinnon, M., Dixon, D.G., 2000. Disease and Gill Lesions in Yellow Perch (*Perca flavescens*) Exposed to Oil Sands Mining-Associated Waters. Ecotox. Environ. Safe. 46, 334-341.

Verbeek, A.G., 1994. A toxicity assessment of oil sands wastewater. MSc Thesis. University of Alberta, Edmonton, AB, Canada.

Videla, P.P., Farwell, A.J., Butler, B.J., Dixon, D.G., 2009. Examining the microbial degradation of naphthenic acids using stable isotope analysis of carbon and nitrogen. Water Air Soil Poll. 197, 107-119.

Watson, J.S., Jones, D.M., Swannell, R.P.J., van Duin, A.C.T., 2002. Formation of carboxylic acids during aerobic biodegradation of crude oil and evidence of microbial oxidation of hopanes. Org. Geochem. 33, 1153-1169.

Wezel, A.P.v., Opperhuizen, A., 1995. Narcosis due to environmental pollutants in aquatic organisms: Residue-based toxicity, mechanisms, and membrane burdens. Crit. Rev. Toxicol. 25, 255-279.

Whitby, C., 2010. Microbial naphthenic acid degradation. Adv. Appl. Microbiol. 70, 93-125.

Yen, T.-W., Marsh, W.P., MacKinnon, M.D., Fedorak, P.M., 2004. Measuring naphthenic acids concentrations in aqueous environmental samples by liquid chromatography. J. Chromatogr. 1033, 83-90.

Young, R.F., Michel, L.M., Fedorak, P.M., 2011. Distribution of naphthenic acids in tissues of laboratory-exposed fish and in wild fishes from near the Athabasca oil sands in Alberta, Canada. Ecotox. Environ. Safe. In Press, Corrected Proof.

Young, R.F., Orr, E.A., Goss, G.G., Fedorak, P.M., 2007. Detection of naphthenic acids in fish exposed to commercial naphthenic acids and oil sands process-affected water. Chemosphere 68, 518-527.

Young, R.F., Wismer, W.V., Fedorak, P.M., 2008. Estimating naphthenic acids concentrations in laboratory-exposed fish and in fish from the wild. Chemosphere 73, 498-505.

Zar, J.H., 1999. Biostatistical analysis. Prentice Hall, New Jersey, NY, USA.

Zhao, Y.H., Yuan, X., Yang, L.H., Wang, L.S., 1996. Quantitative structure-activity relationships of organic acids and bases. B. Environ. Contam. Tox. 57, 242-249.

Zou, L., Han, B., Yan, H., Kasperski, K.L., Xu, Y., Hepler, L.G., 1997. Enthalpy of adsorption and isotherms for adsorption of naphthenic acid onto clays. J. Colloid. Interf. Sci. 190, 472-475.

Zubot, W.A., 2010. Removal of naphthenic acids from oil sands process water using petroleum coke. MSc Thesis. University of Alberta, Edmonton, AB, Canada.

APPENDIX A: GENERAL WATER CHEMISTRY, AND MAJOR ION AND TRACE ELEMENT CONCENTRATIONS

Table A.1. General water chemistry measurements (mean ± SD) for output water samples collected from the simulated wetland microcosms during the 52-week study period. Results are presented separately for each of the 12 treatment scenarios. Sample size (*n*) is presented in parenthesis.

	Treatment Scenario			
Water Chemistry Parameter	Long HRT (400-day)	Long HRT (400-day) Nutrients	Short HRT (40-day)	Short HRT (40-day) Nutrients
Syncrude OSPW				
Dissolved Oxygen (mg/L)	5 ± 1 (48)	5 ± 1 (48)	5 ± 1 (48)	5 ± 1 (48)
Temperature (°C)	26 ± 1 (48)	26 ± 1 (48)	27 ± 1 (48)	26 ± 1 (48)
Conductivity (µS/cm)	2848 ± 365 (48)	2990 ± 431 (48)	3501 ± 328 (48)	3492 ± 341 (48)
pH	8.9 ± 0.1 (48)	8.9 ± 0.2 (48)	8.8 ± 0.2 (48)	8.8 ± 0.3 (48)
Ammonia (mg/L as N)	< 0.01 (27)	< 0.01 (27)	< 0.01 (27)	< 0.01 (27)
Hardness (mg/L as $CaCO_3$)	56 ± 9 (48)	57 ± 7 (48)	76 ± 9 (48)	77 ± 6 (48)
Alkalinity (mg/L as $CaCO_3$)	591 ± 92 (48)	626 ± 104 (48)	739 ± 89 (48)	7387 ± 80 (48)
Suncor OSPW				
Dissolved Oxygen (mg/L)	5 ± 1 (48)	5 ± 1 (48)	5 ± 1 (48)	5 ± 1 (48)
Temperature (°C)	26 ± 1 (48)	26 ± 1 (48)	26 ± 1 (48)	26 ± 1 (48)
Conductivity (µS/cm)	1484 ± 167 (48)	1434 ± 252 (48)	2012 ± 265 (48)	1902 ± 314 (48)
pH	8.7 ± 0.1 (48)	8.7 ± 0.1 (48)	8.7 ± 0.3 (48)	8.7 ± 0.3 (48)
Ammonia (mg/L as N)	< 0.01 (27)	< 0.01 (27)	< 0.01 (27)	< 0.01 (27)
Hardness (mg/L as $CaCO_3$)	53 ± 8 (48)	52 ± 10 (48)	108 ± 25 (48)	100 ± 28 (48)
Alkalinity (mg/L as $CaCO_3$)	423 ± 66 (48)	412 ± 85 (48)	544 ± 107 (48)	512 ± 113 (48)

OSPW = oil sands process-affected waters.
HRT = hydraulic retention time.

Table A.1 continued

Water Chemistry Parameter	Treatment Scenario: Long HRT (400-day)	Long HRT (400-day) Nutrients	Short HRT (40-day)	Short HRT (40-day) Nutrients
Positive Control				
Dissolved Oxygen (mg/L)	5 ± 1 (36)	5 ± 1 (36)	n/a	n/a
Temperature (°C)	25 ± 1 (36)	25 ± 1 (36)	n/a	n/a
Conductivity (µS/cm)	534 ± 69 (36)	561 ± 68 (36)	n/a	n/a
pH	8.0 ± 0.2 (36)	8.0 ± 0.2 (36)	n/a	n/a
Ammonia (mg/L as N)	< 0.01 (18)	< 0.01 (18)	n/a	n/a
Hardness (mg/L as $CaCO_3$)	67 ± 17 (36)	80 ± 16 (36)	n/a	n/a
Alkalinity (mg/L as $CaCO_3$)	79 ± 16 (36)	93 ± 20 (36)	n/a	n/a
Negative Control				
Dissolved Oxygen (mg/L)	5 ± 1 (48)	5 ± 1 (48)	n/a	n/a
Temperature (°C)	25 ± 1 (48)	26 ± 1 (48)	n/a	n/a
Conductivity (µS/cm)	321 ± 44(48)	281 ± 34 (48)	n/a	n/a
pH	7.8 ± 0.3 (48)	7.7 ± 0.3 (48)	n/a	n/a
Ammonia (mg/L as N)	< 0.01 (27)	< 0.01 (27)	n/a	n/a
Hardness (mg/L as $CaCO_3$)	59 ± 8 (48)	47 ± 9 (48)	n/a	n/a
Alkalinity (mg/L as $CaCO_3$)	49 ± 12 (48)	52 ± 11 (48)	n/a	n/a

HRT = hydraulic retention time.
n/a = not available (no control treatments were maintained under short hydraulic retention times).

Table A.2. Major ion concentrations (mean ± SD) for output water samples collected from the simulated wetland microcosms during the 52-week study period. Results are presented separately for each of the 12 treatment scenarios. Sample size (*n*) is presented in parenthesis.

Major Ion	Treatment Scenario			
	Long HRT (400-day)	Long HRT (400-day) Nutrients	Short HRT (40-day)	Short HRT (40-day) Nutrients
Syncrude OSPW				
Na^+ (mg/L)	526 ± 61 (13)	567 ± 53 (13)	638 ± 42 (13)	627 ± 43 (13)
K^+ (mg/L)	6 ± 0 (13)	6 ± 0 (13)	8 ± 1 (13)	8 ± 1 (13)
Mg^{2+} (mg/L)	6 ± 1 (13)	6 ± 0 (13)	10 ± 1 (13)	10 ± 1 (13)
Ca^{2+} (mg/L)	15 ± 2 (13)	16 ± 1 (13)	17 ± 1 (13)	15 ± 3 (13)
Cl^- (mg/L)	211 ± 22 (8)	220 ± 13 (8)	219 ± 11 (8)	211 ± 8 (8)
SO_4^{2-} (mg/L)	180 ± 36 (8)	214 ± 26 (8)	267 ± 17 (8)	254 ± 24 (8)
CO_3^{2-} (mg/L)	35 ± 9 (12)	48 ± 12 (12)	56 ± 17 (9)	58 ± 20 (9)
HCO_3^{2-} (mg/L)	773 ± 81 (12)	795 ± 66 (12)	904 ± 65 (9)	888 ± 58 (9)
Suncor OSPW				
Na^+ (mg/L)	251 ± 33 (13)	245 ± 45 (13)	334 ± 46 (13)	321 ± 44 (13)
K^+ (mg/L)	9 ± 1 (13)	8 ± 2 (11)	12 ± 3 (13)	12 ± 3 (13)
Mg^{2+} (mg/L)	5 ± 1 (13)	6 ± 1 (13)	14 ± 4 (13)	13 ± 4 (13)
Ca^{2+} (mg/L)	12 ± 2 (13)	13 ± 3 (13)	21 ± 7 (13)	21 ± 6 (13)
Cl^- (mg/L)	26 ± 4 (8)	25 ± 5 (8)	30 ± 8 (8)	25 ± 5 (8)
SO_4^{2-} (mg/L)	70 ± 22 (8)	77 ± 30 (8)	123 ± 22 (8)	122 ± 21 (8)
CO_3^{2-} (mg/L)	17 ± 8 (12)	16 ± 11 (12)	39 ± 21 (9)	33 ± 11 (9)
HCO_3^{2-} (mg/L)	538 ± 89 (12)	528 ± 112 (12)	707 ± 145 (9)	672 ± 142 (9)

OSPW = oil sands process-affected waters.
HRT = hydraulic retention time.

Table A.2 continued

Major Ion	Treatment Scenario: Long HRT (400-day)	Long HRT (400-day) Nutrients	Short HRT (40-day)	Short HRT (40-day) Nutrients
Positive Control				
Na^+ (mg/L)	55 ± 3 (15)	53 ± 6 (15)	n/a	n/a
K^+ (mg/L)	< 0.1 (7)	< 0.1 (7)	n/a	n/a
Mg^{2+} (mg/L)	11 ± 2 (15)	11 ± 2 (15)	n/a	n/a
Ca^{2+} (mg/L)	13 ± 5 (15)	20 ± 3 (15)	n/a	n/a
Cl^- (mg/L)	42 ± 3 (10)	42 ± 4 (10)	n/a	n/a
SO_4^{2-} (mg/L)	53 ± 15 (10)	54 ± 16 (10)	n/a	n/a
CO_3^{2-} (mg/L)	< 0.1 (14)	< 0.1 (11)	n/a	n/a
HCO_3^{2-} (mg/L)	100 ± 15 (14)	124 ± 11 (11)	n/a	n/a
Negative Control				
Na^+ (mg/L)	23 ± 3 (13)	22 ± 4 (13)	n/a	n/a
K^+ (mg/L)	< 0.1 (8)	< 0.1 (8)	n/a	n/a
Mg^{2+} (mg/L)	9 ± 1 (13)	8 ± 1 (13)	n/a	n/a
Ca^{2+} (mg/L)	10 ± 3 (13)	9 ± 3 (13)	n/a	n/a
Cl^- (mg/L)	5 ± 3 (8)	4 ± 4 (8)	n/a	n/a
SO_4^{2-} (mg/L)	53 ± 14 (8)	40 ± 10 (8)	n/a	n/a
CO_3^{2-} (mg/L)	< 0.1 (9)	< 0.1 (9)	n/a	n/a
HCO_3^{2-} (mg/L)	59 ± 9 (9)	65 ± 6 (9)	n/a	n/a

HRT = hydraulic retention time.
n/a = not available (no control treatments were maintained under short hydraulic retention times).

Table A.3. Trace element concentrations (mean ± SD) for output water samples collected from the simulated wetland microcosms during the 52-week study period. Results are presented separately for each of the 12 treatment scenarios. Sample size (*n*) is presented in parenthesis.

	Treatment Scenario			
Trace Element	Long HRT (400-day)	Long HRT (400-day) Nutrients	Short HRT (40-day)	Short HRT (40-day) Nutrients
Syncrude OSPW				
Al (mg/L)	0.2 ± 0.0 (13)	0.2 ± 0.0 (13)	BDL	BDL
B (mg/L)	1.5 ± 0.1 (13)	1.7 ± 0.1 (13)	2.2 ± 0.2 (13)	2.1 ± 0.2 (13)
Ba (mg/L)	BDL	BDL	BDL	BDL
Cd (mg/L)	BDL	BDL	BDL	BDL
Co (mg/L)	BDL	BDL	BDL	BDL
Cr (mg/L)	BDL	BDL	BDL	BDL
Cu (mg/L)	BDL	BDL	BDL	BDL
Fe (mg/L)	0.8 ± 0.1 (13)	0.7 ± 0.1 (13)	0.2 ± 0.2 (13)	0.2 ± 0.1 (13)
Li (mg/L)	BDL	BDL	BDL	BDL
Mn (mg/L)	BDL	BDL	BDL	BDL
Mo (mg/L)	BDL	BDL	BDL	BDL
Ni (mg/L)	BDL	BDL	BDL	BDL
P (mg/L)	1.1 ± 0.3 (13)	1.1 ± 0.2 (13)	0.3 ± 0.2 (13)	0.5 ± 0.1 (13)
Pb (mg/L)	BDL	BDL	BDL	BDL
S (mg/L)	61.7 ± 10.1 (13)	72.5 ± 7.3 (13)	97.4 ± 5.8 (13)	92.8 ± 8.2 (13)
Sb (mg/L)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)
Se (mg/L)	BDL	BDL	BDL	BDL
Si (mg/L)	2.7 ± 2.6 (13)	2.8 ± 1.8 (13)	3.3 ± 2.4 (13)	4.8 ± 2.3 (13)
Sr (mg/L)	0.2 ± 0.0 (13)	0.2 ± 0.0 (13)	0.3 ± 0.0 (13)	0.3 ± 0.1 (13)
Ti (mg/L)	BDL	BDL	BDL	BDL
V (mg/L)	BDL	BDL	BDL	BDL
Zn (mg/L)	BDL	BDL	BDL	BDL
Zr (mg/L)	BDL	BDL	BDL	BDL

OSPW = oil sands process-affected waters.
HRT = hydraulic retention time.
BDL = below detection limit.

Table A.3 continued

Trace Element	Treatment Scenario: Long HRT (400-day)	Long HRT (400-day) Nutrients	Short HRT (40-day)	Short HRT (40-day) Nutrients
Suncor OSPW				
Al (mg/L)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	0.1 ± 0.1 (13)	0.1 ± 0.1 (13)
B (mg/L)	1.5 ± 0.2 (13)	1.5 ± 0.2 (13)	2.4 ± 0.3 (13)	2.3 ± 0.3 (13)
Ba (mg/L)	BDL	BDL	BDL	BDL
Cd (mg/L)	BDL	BDL	BDL	BDL
Co (mg/L)	BDL	BDL	BDL	BDL
Cr (mg/L)	BDL	BDL	BDL	BDL
Cu (mg/L)	BDL	BDL	BDL	BDL
Fe (mg/L)	0.7 ± 0.1 (13)	0.7 ± 0.1 (13)	0.3 ± 0.2 (13)	0.2 ± 0.2 (13)
Li (mg/L)	BDL	BDL	BDL	BDL
Mn (mg/L)	BDL	BDL	BDL	BDL
Mo (mg/L)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	0.2 ± 0.1 (13)	0.2 ± 0.1 (13)
Ni (mg/L)	BDL	BDL	BDL	BDL
P (mg/L)	0.6 ± 0.1 (13)	0.7 ± 0.2 (13)	0.2 ± 0.2 (13)	0.2 ± 0.2 (13)
Pb (mg/L)	BDL	BDL	BDL	BDL
S (mg/L)	26.8 ± 7.3 (13)	28.1 ± 8.4 (13)	52.7 ± 15.3 (13)	52.1 ± 16.0 (13)
Sb (mg/L)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)
Se (mg/L)	BDL	BDL	BDL	BDL
Si (mg/L)	1.4 ± 1.8 (13)	3.5 ± 2.9 (13)	3.7 ± 1.5 (13)	2.9 ± 1.6 (13)
Sr (mg/L)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	0.4 ± 0.1 (13)	0.4 ± 0.1 (13)
Ti (mg/L)	BDL	BDL	BDL	BDL
V (mg/L)	BDL	BDL	BDL	BDL
Zn (mg/L)	BDL	BDL	BDL	BDL
Zr (mg/L)	BDL	BDL	BDL	BDL

OSPW = oil sands process-affected waters.
HRT = hydraulic retention time.
BDL = below detection limit.

Table A.3 continued

Trace Element	Treatment Scenario: Long HRT (400-day)	Long HRT (400-day) Nutrients	Short HRT (40-day)	Short HRT (40-day) Nutrients
Positive Control				
Al (mg/L)	BDL	BDL	n/a	n/a
B (mg/L)	BDL	BDL	n/a	n/a
Ba (mg/L)	BDL	BDL	n/a	n/a
Cd (mg/L)	BDL	BDL	n/a	n/a
Co (mg/L)	BDL	BDL	n/a	n/a
Cr (mg/L)	BDL	BDL	n/a	n/a
Cu (mg/L)	BDL	BDL	n/a	n/a
Fe (mg/L)	0.1 ± 0.1 (15)	BDL	n/a	n/a
Li (mg/L)	BDL	BDL	n/a	n/a
Mn (mg/L)	BDL	BDL	n/a	n/a
Mo (mg/L)	BDL	BDL	n/a	n/a
Ni (mg/L)	BDL	BDL	n/a	n/a
P (mg/L)	BDL	0.1 ± 0.1 (15)	n/a	n/a
Pb (mg/L)	BDL	BDL	n/a	n/a
S (mg/L)	17.5 ± 5.1 (15)	16.2 ± 6.1 (15)	n/a	n/a
Sb (mg/L)	0.1 ± 0.0 (15)	0.1 ± 0.0 (15)	n/a	n/a
Se (mg/L)	BDL	BDL	n/a	n/a
Si (mg/L)	3.1 ± 1.5 (15)	1.9 ± 1.0 (15)	n/a	n/a
Sr (mg/L)	0.1 ± 0.0 (15)	0.2 ± 0.0 (15)	n/a	n/a
Ti (mg/L)	BDL	BDL	n/a	n/a
V (mg/L)	BDL	BDL	n/a	n/a
Zn (mg/L)	BDL	BDL	n/a	n/a
Zr (mg/L)	BDL	BDL	n/a	n/a

HRT = hydraulic retention time.
BDL = below detection limit.
n/a = not available (no control treatments were maintained under short hydraulic retention times).

Table A.3 continued

Trace Element	Treatment Scenario: Long HRT (400-day)	Long HRT (400-day) Nutrients	Short HRT (40-day)	Short HRT (40-day) Nutrients
Negative Control				
Al (mg/L)	BDL	BDL	n/a	n/a
B (mg/L)	BDL	BDL	n/a	n/a
Ba (mg/L)	BDL	BDL	n/a	n/a
Cd (mg/L)	BDL	BDL	n/a	n/a
Co (mg/L)	BDL	BDL	n/a	n/a
Cr (mg/L)	BDL	BDL	n/a	n/a
Cu (mg/L)	BDL	BDL	n/a	n/a
Fe (mg/L)	0.1 ± 0.1 (13)	0.2 ± 0.1 (13)	n/a	n/a
Li (mg/L)	BDL	BDL	n/a	n/a
Mn (mg/L)	BDL	BDL	n/a	n/a
Mo (mg/L)	BDL	BDL	n/a	n/a
Ni (mg/L)	BDL	BDL	n/a	n/a
P (mg/L)	BDL	BDL	n/a	n/a
Pb (mg/L)	BDL	BDL	n/a	n/a
S (mg/L)	16.4 ± 5.0 (13)	12.2 ± 3.4 (13)	n/a	n/a
Sb (mg/L)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	n/a	n/a
Se (mg/L)	BDL	BDL	n/a	n/a
Si (mg/L)	0.9 ± 1.1 (13)	1.9 ± 1.9 (13)	n/a	n/a
Sr (mg/L)	0.1 ± 0.0 (13)	0.1 ± 0.0 (13)	n/a	n/a
Ti (mg/L)	BDL	BDL	n/a	n/a
V (mg/L)	BDL	BDL	n/a	n/a
Zn (mg/L)	BDL	BDL	n/a	n/a
Zr (mg/L)	BDL	BDL	n/a	n/a

HRT = hydraulic retention time.
BDL = below detection limit.
n/a = not available (no control treatments were maintained under short hydraulic retention times).

APPENDIX B: ORGANICS ANALYSIS

Table B.1. Mean concentrations of polycyclic aromatic hydrocarbons (PAHs) and benzene, toluene, ethylbenzene, xylene (BTEX) present in input OSPW from Syncrude Canada Ltd. and Suncor Energy Inc. used to charge and recharge the microcosms in the simulated wetland microcosm study (*n* =2 for each OSPW type). Analysis was performed by SRC Analytical (Saskatoon, SK, Canada) using gas chromatography mass spectrometry (GC-MS).

Organic Analyte	Detection Limit (µg/L)	Syncrude OSPW (µg/L)	Suncor OSPW (µg/L)
Benzene	0.2	BDL	BDL
Ethylbenzene	0.2	BDL	BDL
Toluene	0.2	BDL	BDL
Xylene	0.2	BDL	BDL
Acenaphthene	0.05	BDL	BDL
Acenaphthylene	0.05	BDL	BDL
Anthracene	0.05	BDL	BDL
Benzo(a)anthracene	0.1	BDL	BDL
Benzo(a)pyrene	0.01	BDL	BDL
Benzo(b)fluoranthene	0.5	BDL	BDL
Benzo(e)pyrene	0.5	BDL	BDL
Benzo(g,h,i)perylene	0.5	BDL	BDL
Benzo(k)fluoranthene	0.5	BDL	BDL
Chrysene	0.1	BDL	BDL
Dibenzo(a,h)anthracene	0.5	BDL	BDL
Fluoranthene	0.05	BDL	BDL
Fluorene	0.05	BDL	BDL
Indeno(1,2,3-c,d)pyrene	0.5	BDL	BDL
Naphthalene	0.05	BDL	BDL
Perylene	0.5	BDL	BDL
Phenanthrene	0.05	BDL	BDL
Pyrene	0.05	BDL	BDL
Hydrocarbons, F (C6-C10)	5	BDL	BDL

OSPW = oil sands process-affected waters.
BDL = below detection limit

APPENDIX C: ION SUPPRESSION IN NAPHTHENIC ACIDS MASS SPECTRA DUE TO DISSOLVED SALTS

The high salinity of OSPW can complicate the mass spectrometric analysis of NAs resulting in analytical error and biases. During analysis, the salts in OSPW or those associated with extracted NAs, can cause ion suppression and detector saturation. Methods used to reduce ion suppression due to the presence of salts, is to dilute the sample and/or use a preparatory clean-up procedure using solid phase extraction (SPE) ENV+ cartridges prior to analysis.

This appendix provides a comparison of the mass spectra generated by the electrospray-ionization mass spectrometry (ESI-MS) analysis of naphthenic acids (NAs) for three un-identified oil sands process-affected water samples (Figures C1, C2, and C3). Each of the samples were analysed using three variations of the analytical technique: direct injection, direct injection with a 1 in 5 dilution; and, injection of the NAs after a solid phase extraction (SPE) clean-up procedure. Three different spectra for each sample are presented showing the differences between the complexity of the spectra depending on the method used. The spectra from direct injection showed a flattened, bimodal peak shape indicating either detector saturation or ion suppression due to the presence of salts. Due to the relatively low peak intensity, high ion suppression was most likely occurring. Evidence of ion suppression, although still present, was minimized in the spectra produced by the 1 in 5 dilution. In addition, the presence of a number of previously indistinguishable high mass, non-NAs peaks became apparent. Sample spectra produced after the SPE clean-up procedure had a good chromatographic peak shape and indicated that no or minimal ion suppression occurred.

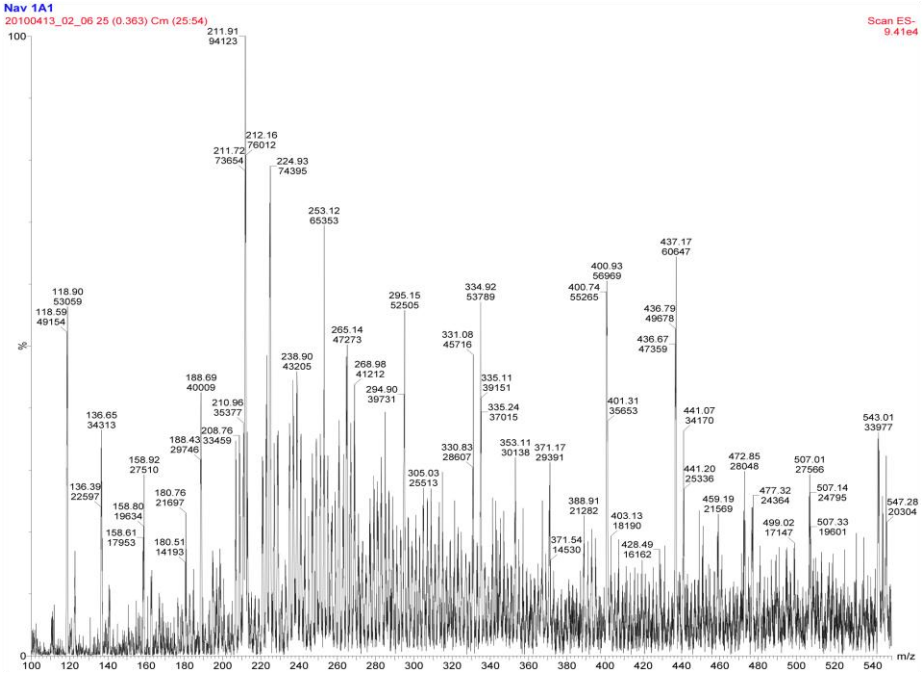
In the mass spectra produced by ESI-MS analysis, ion suppression or detector saturation due to the presence of salts, can lead to poor chromatographic peak shape (e.g., flattened and bimodal as opposed to unimodal peak typical of OSPW NAs), and obscure the detection of high mass non-NAs peaks, which if un-accounted for can lead to erroneously high total NAs concentration measurements. Differences in the complexity of mass spectra that are produced during NAs analysis depend on the nature of the preparatory steps used, or not used, making it difficult to assess the composition of a given NAs mixture and compare results among various studies.

Mass spectra were kindly produced and provided by Brian Fahlman, Kerry Peru, and John Headley of Environment Canada (Saskatoon, SK). The spectra were interpreted with the assistance of Brian Fahlman.

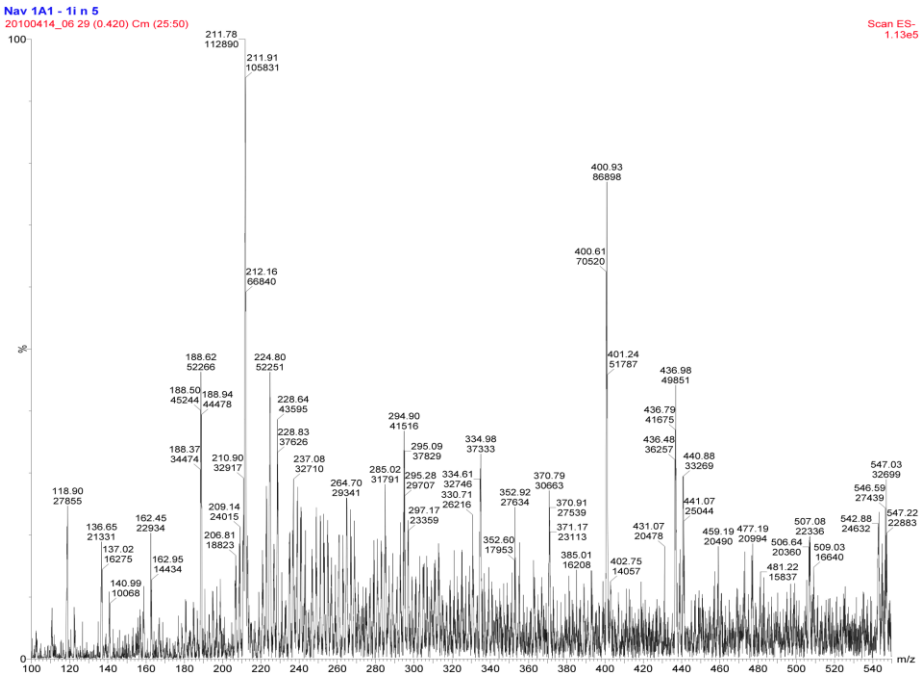
Figure C1. Mass spectra of NAs in an unidentified oil sands process-affected water sample (Sample 1A1) analyzed using electrospray ionization mass spectrometry (ESI-MS) via (a) direct injection, (b) direct injection with a 1 in 5 dilution, and (c) injection of the NAs after a solid phase extraction (SPE) clean-up procedure.

Sample 1A1

(a) Direct injection



(b) Direct injection with a 1 in 5 dilution



(c) Injection after solid phase extraction (SPE) clean-up

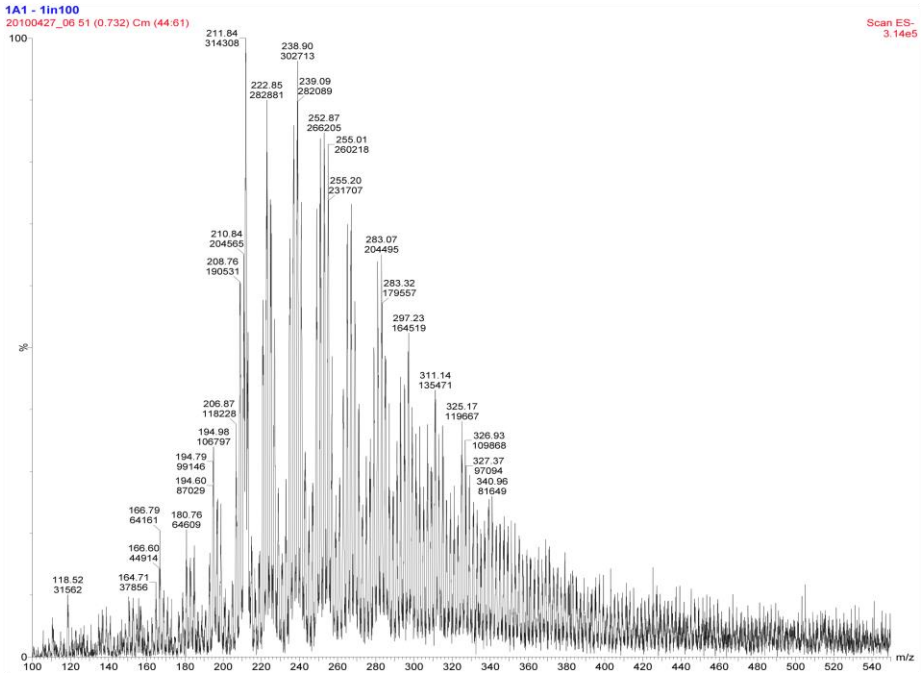
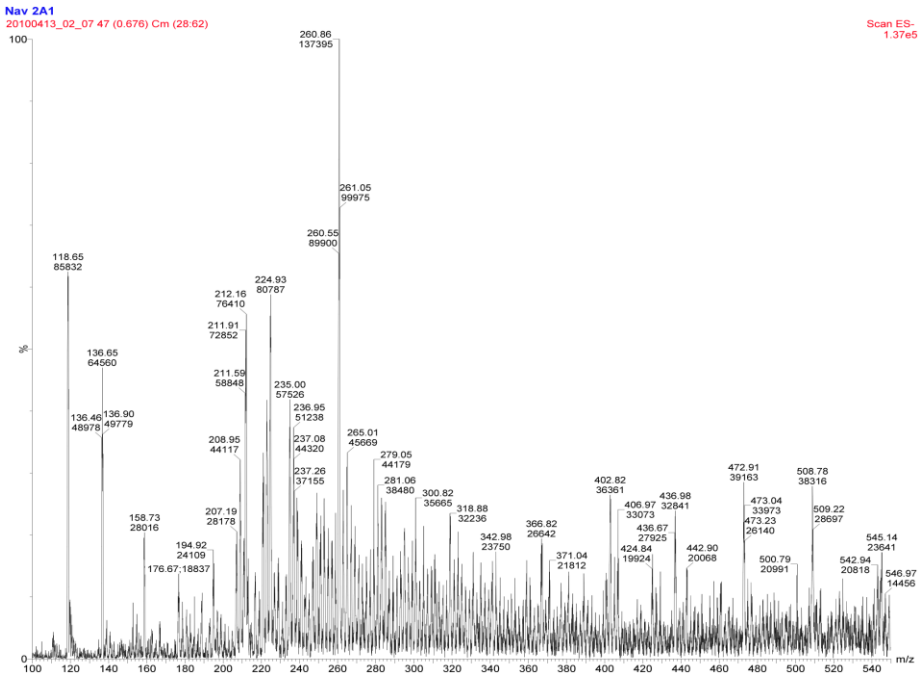


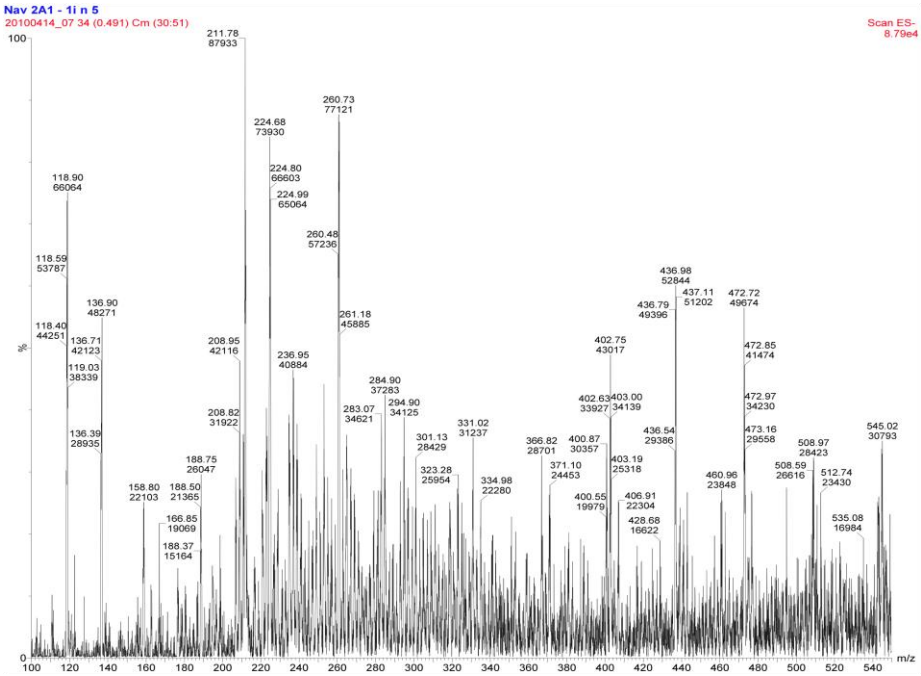
Figure C2. Mass spectra of NAs in an unidentified oil sands process-affected water sample (Sample 2A1) analyzed using electrospray ionization mass spectrometry (ESI-MS) via (a) direct injection, (b) direct injection with a 1 in 5 dilution, and (c) injection of the NAs after a solid phase extraction (SPE) clean-up procedure.

Sample 2A1

(a) Direct injection



(b) Direct injection with a 1 in 5 dilution



c) Injection after solid phase extraction (SPE) clean-up

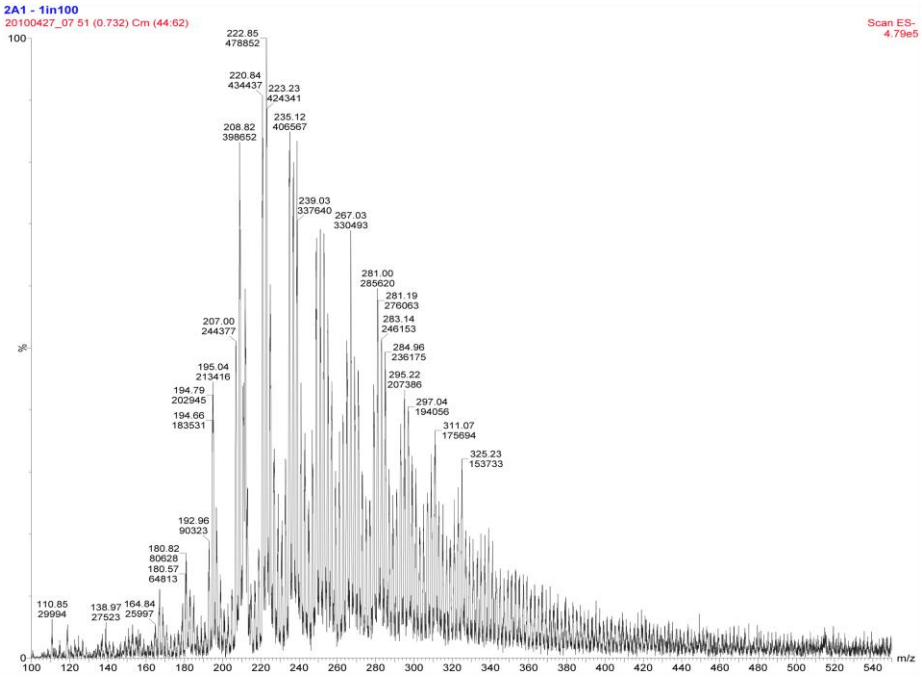
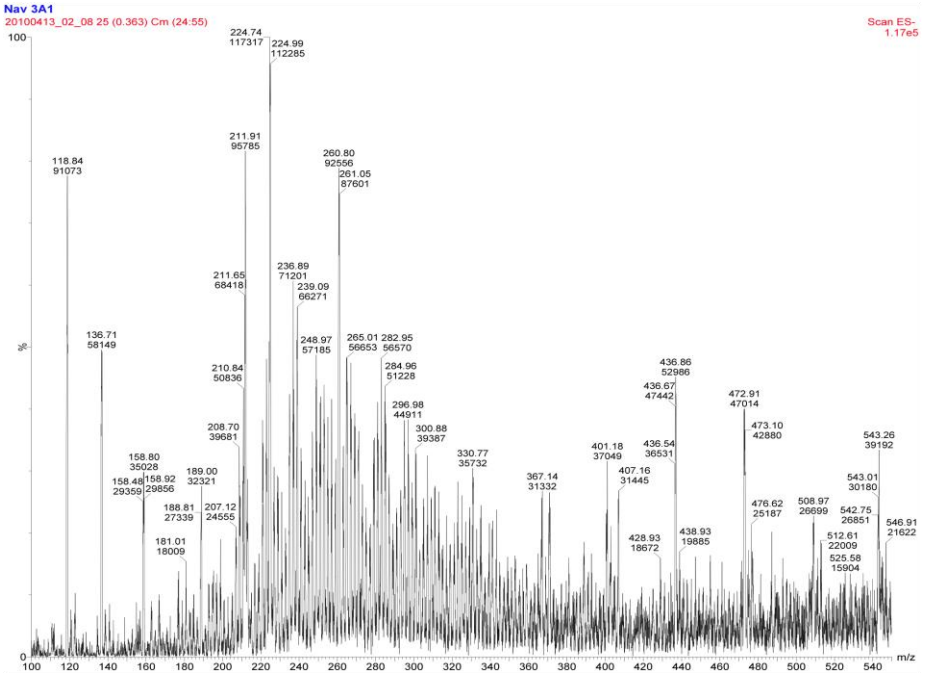


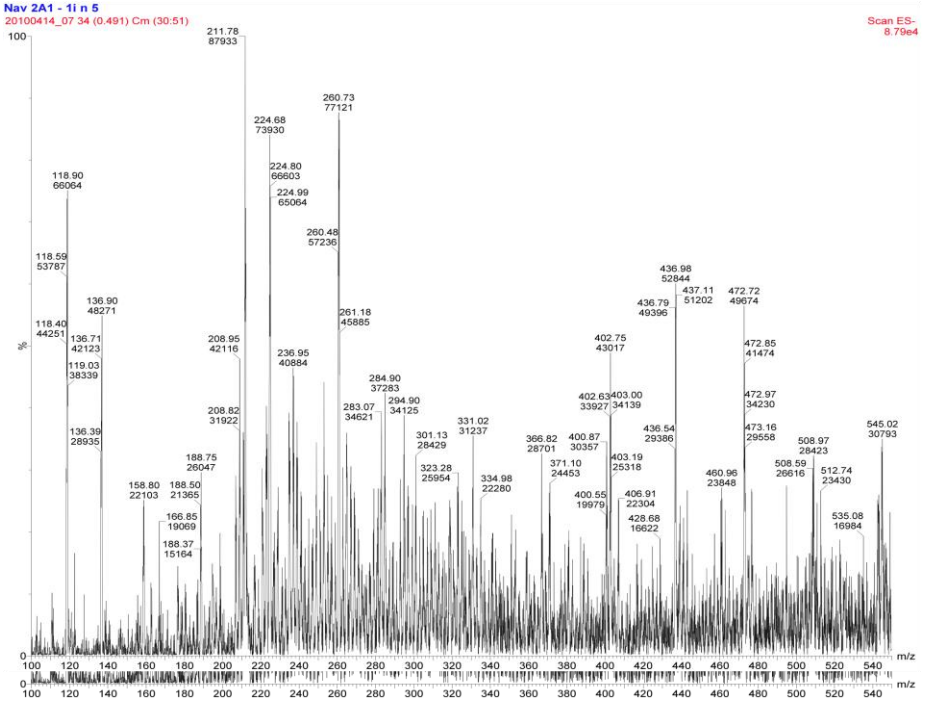
Figure C3. Mass spectra of NAs in an unidentified oil sands process-affected water sample (Sample 3A1) analyzed using electrospray ionization mass spectrometry (ESI-MS) via (a) direct injection, (b) direct injection with a 1 in 5 dilution, and (c) injection of the NAs after a solid phase extraction (SPE) clean-up procedure.

Sample 3A1

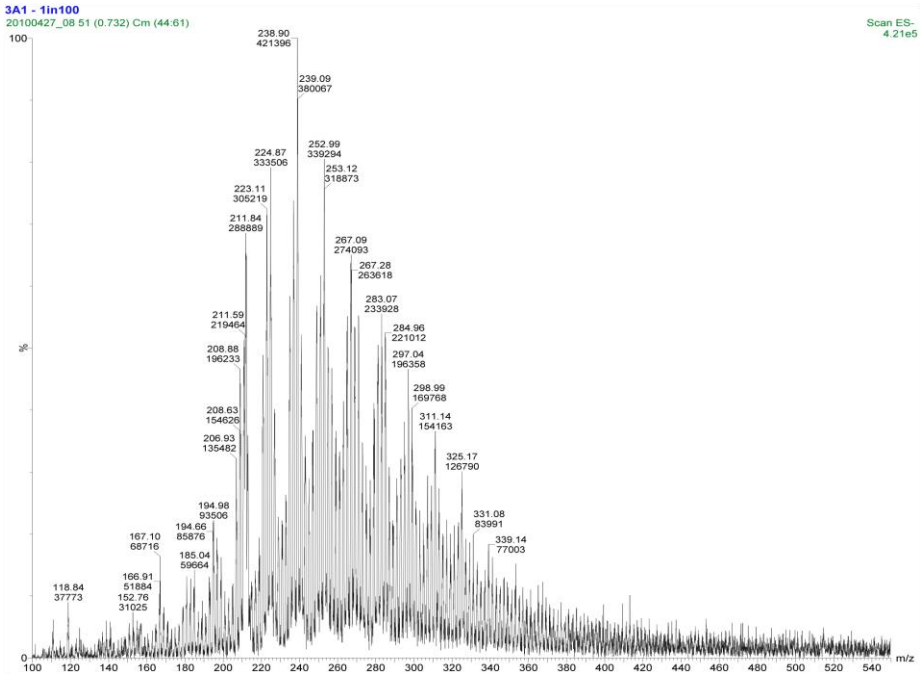
(a) Direct injection



(b) Direct injection with a 1 in 5 dilution



c) Injection after solid phase extraction (SPE) clean-up



APPENDIX D: NAPHTHENIC ACIDS FRACTIONATION

D.1 Introduction

Field observations at the Athabasca oil sands (AOS) suggest that naphthenic acids (NAs) contained in OSPW retention ponds degrade under natural conditions, however, the process is quite slow and incomplete (Holowenko et al., 2002). Complete microbial mineralization does not occur and a portion of the NAs mixture, particularly the heavier molecular weight NAs, persist (Quagraine et al., 2005a). Even after a period of 7 to 11 years, with no subsequent input of tailings water, the total concentration of NAs in some ponds had not decreased below 20 mg/L (Leung et al., 2001; Holowenko et al., 2002). Herman et al. (1994) demonstrated that mineralization of oil sands NAs, via microbial activity, was possible and that this corresponded to a reduction in Microtox® acute toxicity. However, they found that residual (i.e., chronic) toxicity persisted and that this could potentially be related to the more persistent NAs. The exact relationship between the persistent NAs and the associated persistent toxicity of OSPW remains unclear. In Chapter 4 of this research, it was demonstrated that the total NAs concentration was not reduced below 20 mg/L in treatments with a long HRT and added nutrients (Figure 4.3 and 4.4), and that the chronic aquatic toxicity remained unchanged as measured using the Microtox® bioassay (EC_{20}s) (Figure 4.6 and 4.7). In Chapter 5, it was hypothesized that clusters A to D, the more readily biodegradable fraction, could be responsible for the observed acute toxicity. Similarly, those clusters that were the least affected by biodegradation (clusters H and I) could be responsible for the persistent toxicity. Further investigation would begin with the separation of a given NAs mixture into select fractions, isolated with sufficient volumes and NAs concentrations, to conduct chronic toxicity bioassays with individual fractions.

Two recent attempts to fractionate OSPW NAs had limited success. Lo et al. (2006) used anion exchange chromatography to separate a NAs mixture based on pK_a differences. The mixture was separated into eight fractions, however, due to incomplete separation and some fractions containing similar congener profiles, only three fractions were considered to be different enough to evaluate toxicologically. Naphthenic acids profiles were characterized by electrospray ionization mass spectrometry (ESI-MS). Those fractions containing a higher proportion of multi-ring structures were less toxic in the Microtox® bioassay (EC_{50}). More recently, Frank et

al. (2008) assessed the toxicity of partially separated fractions of OSPW NAs also using the Microtox® bioassay (EC_{50}). The NAs were first methylated and then distilled based on differences in boiling points using a Kugelrohr distillation apparatus. Stepped distillation temperatures of 130, 160, 190, and 220°C were held constant for 20 minutes and the methylated NAs for each fraction were condensed in a collection vessel over ice water. The five fractions (130, 160, 190, 220, and >220°C residue) were de-methylated prior to an assessment of toxicity using the Microtox® bioassay (EC_{50}). The results showed that the lowest molecular weight NAs (130°C) had higher toxic potency than the highest molecular weight NAs (>220°C), although there was some reversal in the toxic responses for the fractions collected at 160, 190, and 220°C. Neither of these studies achieved complete separation of the NAs mixture (i.e., there were overlapping NAs congeners in the fractions compared) or assessed chronic toxicity.

In order to test the hypothesis that the more persistent components of an OSPW NAs mixture (e.g., NAs congeners with higher carbon numbers and degrees of cyclization) may be responsible for the observed residual chronic toxicity identified in Chapters 4 and 5, this study first attempted to fractionate an OSPW NAs before the toxicological properties associated with select fractions could be assessed.

D.2 Materials and Methods

The separation of OSPW NAs was attempted using two methods: off-line anion-exchange chromatography in glass columns; and, batch-wise filtration and elution with a proprietary co-polymer obtained from professor L. Wilson, Department of Chemistry, University of Saskatchewan (Saskatoon, SK).

D.2.1 Offline Anion-Exchange Chromatography

Syncrude OSPW was collected and shipped to the Toxicology Centre, University of Saskatchewan (Saskatoon, SK) from Syncrude Canada Ltd.'s lease site at the AOS on June 29, 2010, where it was stored at 4°C until experimentation. This preliminary investigation was conducted to determine the NAs extraction efficiency using both the weakly basic DOWEX™ 66 (Dowex-66), and the strongly basic DOWEX™ 1×2-200 (Dowex-200) ion-exchange resins (DOW Chemical Co.) obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON). The free base

form of Dowex-66 and the Cl form of Dowex-200 have exchange capacities of 1.47 meq/mL and 0.6 meq/mL, respectively. Columns were made of borosilicate glass and were 20 cm in length with an inner diameter of 1 cm.

D.2.1.1 Resin and Column Preparation

Initially, 40 g of Dowex-66, and 60 g of Dowex-200 (dry weight) were weighed and wetted. Approximately 150 mL of de-ionized Barnstead water was added to each resin in a 250-ml glass beaker and swirled for 1 to 2 minutes. The resins in the beakers were then allowed to settle for 15 minutes, after which time the water was decanted and the procedure repeated a total of three times. The charging, exercising, and conditioning of the resins used slightly different procedures according to their respective original forms (e.g., free base and Cl forms). The Dowex-66 resin and de-ionized Barnstead water slurry was poured into the column having an approximate bed volume (BV) of 20 mL. The column was then rinsed with 50 mL of 1 N NaOH, under gravity, four times. The Dowex-200 resin and water slurry was loaded onto a second column. However, the resin inside the column was exercised with 50 mL of 5% HCl, under gravity, three times. To remove the acid, the column was then rinsed with 250 mL of de-ionized Barnstead water in five, 50 mL increments. The resin was then converted to the NaOH form by rinsing it with 50 mL of NaOH a total of five times. Both columns were subsequently rinsed with approximately 500 mL of de-ionized Barnstead water to remove excess NaOH until the pH and conductivity measurements of the rinsate were below the 0.01% values of the 1 N NaOH solution.

D.2.1.2 Experimental Procedure and Analysis

An OSPW sample (100 mL) was poured into a glass 250-mL separatory funnel connected to the top of a 20 cm glass column. The contact time between OSPW and the resin inside the column was controlled by gravity and a valve at the bottom of the column. The drip rate was set to approximately 30 drops per minute, which required a total of 45 minutes for the 100-mL OSPW sample to pass through the column and be collected into a 125-mL amber glass bottle. The void volume of the column was estimated to be 10 mL, diluting the collected fraction by approximately 10%. After the first 100 mL output OSPW was collected (sample B), the experiment was continued by passing a second 100-mL OSPW sample through the column. The second 100-mL fraction was collected and labelled as sample C. The input sample from the

stock OSPW supply was labelled as sample A. At this point, NAs in OSPW should be bound to the anionic resin in the column. Subsequent elutions were conducted using 125 mL of de-ionized Barnstead water, 125 mL of pH =4 water, 125 mL of pH =2 water, and 125 mL of pH =2 water and 50% methanol solution. Sample fractions were collected between each elution step and labelled D through G, respectively.

A 20-mL sub-sample of the input OSPW (sample A), and the first and second 100-mL output samples (samples B and C) were analyzed using the Microtox® bioassay. Total NAs concentrations were measured in all samples using electrospray-ionization mass spectrometry (ESI-MS) by Environment Canada (Saskatoon, SK) according to their standard methods (Headley et al., 2002a). Inorganic cation and anion analysis of the collected sample fractions were measured following EPA Method 300.1 and employed a Dionex ICS-3000 dual Ion Chromatography system (Dionex, Sunnyvale, CA). Prior to analysis, water samples were filtered through a 0.45-μm membrane filter. The software Chromeleon 6.80 was used to simultaneously determine cation and anion concentrations.

D.2.2 Batch-Wise Co-Polymer Fractionation

The Syncrude OSPW used in this part of the study was the same OSPW used in the previous column chromatography experiment. Three slightly different proprietary resin co-polymers labelled C1, C2, and C3, were obtained from Professor L. Wilson, Department of Chemistry, University of Saskatchewan (Saskatoon, SK). Information regarding the co-polymers was proprietary, but they were expected to separate the NAs mixture based on polarity and size. As before, NAs analysis was conducted using ESI-MS analysis by Environment Canada. However, all steps for the accurate quantification of NAs were not taken, thus results for total NAs concentrations are not presented. The primary objective was to characterize the changes in the fractional abundance of NAs congeners due to co-polymer separation, and so only the results of the fingerprinting analysis were compared in detail.

D.2.2.1 Experimental Protocol

The following protocol was applied to the three different co-polymers (C1, C2, and C3) in order to capture NAs in each co-polymer and then selectively remove the captured NAs using solutions

with different percent concentrations (v/v) of methanol and Millipore water. The OSPW was first filtered to remove any suspended material using Whatman No. 2 cellulose filter paper with a pore size of 8 μm. One gram of the co-polymer was added to a 2-L glass beaker containing 1 L of filtered OSPW. The mixture was stirred for 24 hours. The co-polymer was then filtered from the OSPW and the filtrate analyzed by mass spectrometry. At this point the NAs should be bound to the co-polymer. Subsequently, the filtered co-polymer was stirred in another 2-L glass beaker with 5% (v/v) methanol and Millipore water for 24 hours. Again, the co-polymer was filtered from the 5% (v/v) methanol solution and the filtrate was subjected to rotary evaporation to remove the methanol and concentrate the NAs for ESI-MS analysis. This procedure was repeated for the same co-polymer using 10% (v/v) methanol and 100% methanol solutions separately. The entire process was repeated for the other two co-polymers.

D.2.2.2 Data Analysis

As described in Chapters 5 and 6, the relative abundances of NAs congeners identified in the three-dimensional fingerprints using ESI-MS were analyzed according to the arbitrarily defined clusters labelled A through I, carbon number (increments of 3, from 6 to 38), and *Z* series (i.e., cyclization). The percent factional abundance for each group of NAs congeners is summarized in separate tables to determine if the distribution patterns were influenced by the different co-polymers and/or washes with increasing methanol concentrations. Due to the nature of the analysis and the inconsistent preparatory work required to isolate and concentrate NAs for ESI-MS analysis, the fractional abundances of the distributions were not assessed statistically.

D.3 Results and Discussion

D.3.1 Anion Exchange Chromatography

The column containing Dowex-200 resin was unsuccessful in this off-line anion exchange column experiment because it did not produce an acceptable output flow rate even with the valve fully open. It appears that under gravity, the extremely fine structure of the resin restricts flow rates to near zero, and would likely require pressure from a pumping system to maintain a constant flow rate. Therefore, only the data from the column containing the Dowex-66 resin are presented. Dowex-66 resin has a larger, macroporous structure, which did not impede the flow of OSPW through the column.

The effectiveness of the Dowex-66 anion exchange resin column was validated based on the anion and cation concentrations of the input OSPW (sample A) and the first 100-mL fraction collected after passing through the column (sample B) (Table D.1). The results show that while anion concentrations within OSPW were drastically reduced after passing through the column, the concentration of the cations were much less affected. Results of the NAs extraction and elution experiment are presented in Table D.2. The total NAs concentration was reduced from 55 mg/L of the input OSPW (sample A) to 2 mg/L (sample B) and 5 mg/L (sample C) after passing through the column. This suggests that the NAs were bound to the Dowex-66 resin with relative ease, and that the binding capacity was not saturated with the subsequent passing of the second 100-mL OSPW fraction.

Table D.1. Anion and cation concentrations (mg/L) in the input OSPW (sample A) and in the ouput OSPW (sample B) after passing through an off-line anion exchange column containing a weakly basic resin (Dowex-66).

	Anion (mg/L)					
Sample	F^-	Cl^-	NO_2^-	Br^-	SO_4^{2-}	NO_3^-
A - Inut OSPW	2.31	256.79	0.20	0.09	291.71	0.06
B - Output OSPW (1st 100 mL)	1.45	22.04	0.01	0.00	0.04	0.01
	Cation (mg/L)					
	Li^+	Na^+	NH_4^+	K^+	Mg^{2+}	Ca^{2+}
A - Inut OSPW	0.08	495.84	4.51	8.21	11.47	17.52
B - Output OSPW (1st 100 mL)	0.07	427.10	2.99	6.40	0.22	7.87

OSPW = oil sands process-affected waters.

Unfortunately, the ability of the strongly basic Dowex-200 anion exchange resin to bind NAs from OSPW was not assessed. However, Gaikar and Maiti (1996) investigated the adsorption of NAs from petroleum oil using both a macroporous weakly anionic ion-exchanger with a weak tertiary amine group (similar to the Dowex-66 resin used in this study), and a strong anionic ion-exchange resin. The authors determined that the weakly basic resin had a higher capacity for, and was more successful at, adsorbing NAs than the strong anionic ion-exchanger. Similarly, the NAs in OSPW were successfully removed by the Dowex-66 resin in this study, due to a

relatively high porosity and increased accessibility of the amine functional group of the resin allowing interaction with NAs via hydrogen bonding (Gaikar and Maiti, 1996). The Microtox® bioassay demonstrated that the toxicity associated with OSPW (EC_{20} =32.4% v/v) was completely removed (EC_{20} >100% v/v) with the sorption of NAs (including other potentially toxic constituents of OSPW) to the resin. This was expected as the removal of >90% of total NAs to a concentration of 5 mg/L is below the toxicity thresholds for not only *Vibrio fischeri* (used in the Microtox® bioassay), but for most aquatic eukaryotic organisms as well.

Table D.2. Total NAs concentrations (mg/L) and Microtox EC_{20} (% v/v) values for samples collected during the off-line chromatography column experiment using the weakly basic, Dowex-66 ion-exchange resin.

Sample	NAs Concentration (mg/L)	Microtox® EC_{20} (% v/v)
A - Inut OSPW	55	32
B - Output OSPW (1st 100 mL)	2	> 100
C - Output OSPW (2nd 100 mL)	5	> 100
D - Barnstead water rinse (125 mL)	BDL	
E - pH = 4 elution (125 mL)	BDL	
F - pH = 2 elution (125 mL)	BDL	
G - pH = 2 plus 50% methanol elution	BDL	

BDL = below detection limit.

Attempts at removing the NAs from the Dowex-66 resin were surprisingly unsuccessful. The de-ionized Barnstead water rinse (sample D) was expected to wash the residual OSPW from the column and therefore, not contain any NAs (Table D.2). However, using the pH =4 and pH =2 elution solutions, NAs adsorbed on to the resin should have been protonated. Both of these elution steps should have washed out at least some of the neutral, non-polar NAs from the column, especially since the pK_a of NAs is between 5 and 6 (Headley and McMartin, 2004). Even the subsequent use of a solution containing 50% methanol with a pH =2 (increased non-polarity to enhance NAs desorption) was unsuccessful at removing NAs. Desorption using methanol concentrations >50% were not attempted due to incompatibility with the ESI-MS

analysis. As a result, this method of attempting NAs fractionation was abandoned in favour of a more promising alternative using a proprietary co-polymer.

D.3.2 Batch-Wise Co-Polymer Fractionation

According to distributions based on the arbitrarily defined clusters A through I, results showed that the NAs distributions in the OSPW filtrate after the initial mixing with co-polymers C1, C2, and C3 were all similar to each other, and with the input OSPW (i.e., prior to mixing with any co-polymers) (Table D.3). This suggests that all NAs adsorbed onto the co-polymers in a consistent manner and without any selectivity. However, some selectivity was observed during the sequential washes with increasing concentrations of methanol. For co-polymer C1, after the wash with 100% methanol, the largest increase in the proportion of NAs congeners desorbed was observed for clusters E and H. The NAs in clusters E and H contain congeners with 14 to 20 carbons and 3 to 6 rings. In addition, co-polymers C1 and C2 demonstrated a selectivity towards the NAs congeners that make up cluster E, for which the majority were desorbed sequentially with washes containing increasing concentrations of methanol. However, the NAs in cluster E were maximally desorbed from co-polymer C3 with the 5% methanol wash, without any further desorption with subsequent washing using increasing methanol concentrations. Co-polymers C1, C2, and C3 appeared to be strongly selective for the most complex NAs congeners (cluster I), whose distribution was unaffected by sequential washes using increasing methanol concentrations. Cluster I contains the heavier molecular weight NAs with highest number of carbons (17 to 20) and highest number of rings (5 to 6 rings). The fractional abundance of the other NAs congeners were largely unaffected by type of co-polymer and increasing methanol concentrations.

Table D.3. Naphthenic acids congener distributions (%) based on clusters A through I using co-polymers C1, C2, and C3, and increasing percent methanol (v/v) concentrations for the batch-wise fractionation experiment.

Cluster	Input OSPW	Co-polymer and Sample Treatment											
		C1 Resin Only	C1 5% MeOH	C1 10% MeOH	C1 100% MeOH	C2 Resin Only	C2 5% MeOH	C2 10% MeOH	C2 100% MeOH	C3 Resin Only	C3 5% MeOH	C3 10% MeOH	C3 100% MeOH
A	15	17	11	11	5	16	20	13	8	15	14	13	13
B	4	4	4	5	3	4	4	5	5	4	5	4	5
C [a]	–	–	–	–	–	–	–	–	–	–	–	–	–
D	26	25	23	24	19	25	23	24	23	25	22	22	26
E	14	13	18	19	27	12	14	18	26	13	18	18	17
F	2	1	1	1	1	1	1	1	1	1	2	2	1
G	17	18	18	16	15	18	17	17	14	19	15	15	13
H	10	10	11	11	16	11	9	10	12	10	10	11	12
I	12	12	14	13	14	13	12	12	11	13	14	15	13

[a]No NAs congeners were detected in cluster C for any of the filtrate samples that were analyzed using electrospray ionization mass spectrometry (ESI-MS).

OSPW = oil sands process-affected waters.
MeOH = methanol.

Table D.4 shows that the NAs distributions, based on carbon number, in the OSPW filtrate after the initial mixing with co-polymers C1, C2, and C3 were all similar to each other and with the input OSPW (i.e., prior to mixing with any co-polymers). As expected, all NAs were adsorbed onto the co-polymers in a consistent manner and without any great degree of selectivity. However, with sequential methanol washes, co-polymer C1 was slightly more selective for carbon number groups 15, 16, 17 and 18, 19, 20 where washing the co-polymer C1 with 100% methanol resulted in their maximum desorption. This was consistent with the results obtained using the cluster analysis, where co-polymer C1 also appeared to bind and maximally desorb NAs congeners containing 14 to 20 carbons. All three co-polymers showed an increasing selectivity, with increasing concentrations of methanol, towards NAs in the carbon number groups 24, 25, 26. However, co-polymer C2 was the most effective at selectively binding NAs present in carbon number groups 24, 25, 26 until they were maximally desorbed with the 100% methanol wash. Again, co-polymer C3 appeared to be the least effective for selective retention of NAs congeners based on carbon number groups.

Table D.4. Naphthenic acids congener distributions (%) based on carbon number groups using co-polymers C1, C2, and C3, and increasing percent methanol (v/v) concentrations for the batch-wise fractionation experiment.

		Co-polymer and Sample Treatment											
Carbon Number Group	Input OSPW	C1 Resin Only	C1 5% MeOH	C1 10% MeOH	C1 100% MeOH	C2 Resin Only	C2 5% MeOH	C2 10% MeOH	C2 100% MeOH	C3 Resin Only	C3 5% MeOH	C3 10% MeOH	C3 100% MeOH
6, 7, 8	1	1	0	1	0	0	0	1	0	1	0	1	0
9, 10, 11	4	4	1	3	1	4	8	5	6	4	3	4	3
12, 13, 14	21	23	22	20	16	22	23	22	17	21	24	21	23
15, 16, 17	29	27	32	29	38	28	29	25	27	27	33	30	31
18, 19, 20	24	24	27	24	27	24	24	23	18	25	26	25	24
21, 22, 23	11	10	11	11	11	10	9	10	8	10	9	10	10
24, 25, 26	4	4	4	6	6	5	6	11	21	4	4	6	7
27, 28, 29	4	4	3	4	1	4	1	2	3	5	0	3	2
30, 31, 32	2	2	0	1	0	2	0	0	0	2	0	0	0
33, 34, 35	0	1	0	1	0	1	0	1	0	1	0	0	0
36, 37, 38	0	0	0	0	0	0	0	0	0	0	0	0	0

OSPW = oil sands process-affected waters.
MeOH = methanol.

Similar to the distribution of NAs congeners based on clusters and carbon number groups, the fractional distribution, based on *Z* series, of the OSPW filtrate after the initial mixing with co-polymers C1, C2, were similar to each other and to the input OSPW. This suggested that the NAs were adsorbed onto the co-polymers with negligible selectivity (Table D.5). In contrast, co-polymer C3 appeared to be slightly less selective than co-polymers C1 and C2 for NAs congeners with zero rings ($Z = 0$) as the filtrate after the initial wash without any methanol contained a slightly higher proportion of NAs within the $Z = 0$ group. During the sequential washing of the three co-polymers with increasing concentrations of methanol, co-polymers C2 appeared to selectively bind NAs congeners in the Z = -6 (3 rings) series with the most success because desorption with increasing methanol concentrations was not as significant as the desorption observed for co-polymers C1 and C3. Co-polymers C1 and C3 also appeared to bind NAs congeners in the $Z = -6$ group series, but as a result of washing with increasing methanol concentrations, these NAs were liberated more readily than those bound to co-polymer C1. All three polymers appeared to bind NAs congeners in the Z = 0 and -2 (straight chain and single ring) groups as increasing concentrations of methanol did not liberate these fractions from the co-polymers. Co-polymer C2 had the highest selectivity for the Z = -12 (6 ring) NAs congeners, which after washing with 100% methanol, resulted in greater desorption of the 6 ring NAs compared to co-polymers C1 and C3.

Table D.5. Naphthenic acids congener distributions (%) based on *Z* series using co-polymers C1, C2, and C3, and increasing percent methanol (v/v) concentrations for the batch-wise fractionation experiment.

		Co-polymer and Sample Treatment											
Z Series	Input OSPW	C1 Resin Only	C1 5% MeOH	C1 10% MeOH	C1 100% MeOH	C2 Resin Only	C2 5% MeOH	C2 10% MeOH	C2 100% MeOH	C3 Resin Only	C3 5% MeOH	C3 10% MeOH	C3 100% MeOH
0	18	19	12	14	11	18	12	14	8	21	13	14	14
-2	23	22	17	17	13	22	24	18	16	21	18	17	18
-4	21	21	25	22	21	21	23	21	17	20	25	22	22
-6	13	13	19	18	25	13	16	16	17	13	19	17	17
-8	9	9	10	10	12	9	8	9	10	9	9	10	10
-10	8	8	8	10	8	8	7	7	8	8	8	9	9
-12	8	8	9	9	10	9	10	15	24	8	8	11	10

OSPW = oil sands process-affected waters.
MeOH = methanol.

D.4 Conclusions

In general, co-polymers C1 and C2 show the most promise for fractionating NAs mixtures. This batch-wise fraction method could be applied to larger volumes of OSPW allowing the concentrations of NAs in separated fractions to be high enough to conduct toxicity bioassays. Although this preliminary exercise did not show complete separation of the NAs mixture, the proof-of-concept experiment was successful at demonstrating the potential for these proprietary co-polymers to separate NAs mixtures using the described methodology. However, without accurate total NAs concentration data, it was difficult to determine the actual change in the fractional abundance of fractionated NAs. Having information on the relative distributions only, it was unclear whether a lack of an observed change in the fractional distribution of NAs was the result of NAs being strongly sorbed onto the co-polymers, or due to a lack of initial loading (i.e., weak sorption) onto the co-polymers in the first place. Future experiments should consider using smaller methanol concentration increments (e.g., 1, 2, 3, 4, 5%, etc). It is suspected that refinements to the methods, the biggest one being pH manipulation, used in combination with sequential methanol washes, would drastically improve separation potentially resulting in the complete separation of an OSPW NAs mixture.

APPENDIX E: MICELLE FORMATION BY NAPHTHENIC ACIDS

E.1 Introduction

Naphthenic acids (NAs) are a mixture of carboxylic acids naturally present in bitumen. These surface active organic acids (i.e., with surfactant properties) play an important role in the Clark hot water extraction process promoting the liberation of bitumen from the sand particles and becoming solubilized in the alkaline tailings water as sodium naphthenates in the process (Schramm et al., 2000; Clemente et al., 2003). Naphthenic acids congeners are similar to natural fatty acids and surfactants in that they contain at least one hydrophilic carboxylic acid "head", and a hydrophobic acyclic and aliphatic hydrocarbon "tail".

Like fatty acids, anionic surfactants present in aqueous solutions can form aggregates to minimize contact between the hydrophobic "tails" and the polar water molecules. In these aggregates, or micelles, the surfactants have oriented themselves with their hydrophobic "tails" directed toward the interior of the micelle and the hydrophilic heads directed outwards to the water (Rosen, 2004). Surfactant molecules dissolved in water will begin to associate into aggregates (i.e., micelles) once a certain concentration is reached. This is commonly referred to as the critical micelle concentration (CMC) and is a property of the surfactant and other factors such as temperature and electrolyte concentrations (Schramm et al., 2000). A low CMC means that micelle formation occurs at a lower surfactant concentration. Micellization is a kinetic process in which there is an equilibrium between the freely dissolved monomers and the micelles. This dynamic system has been summarized by Tadros (2000) using the equation $n\mathrm{S} \leftrightarrows \mathrm{M}$, where n is the number of single surfactant monomers, S, that associate to form a micelle, M. Any additional increase in the concentration of the surfactant monomers will tend to form micelles and the concentration of the monomer units remains relatively constant above the CMC (Tadros, 2000).

It was hypothesized that the oil sands process-affected water (OSPW) NAs, having a greater proportion of heavier molecular weight NAs with multiple rings (Holowenko et al., 2002; Scott et al., 2005), have the ability to form micelles that are more stable in an aqueous environment. In contrast, commercial NAs having smaller molecular weights and simpler conformations with

shorter carbon chains and fewer rings, may not have, or have a reduced, ability to form micelles. The longer carbon chains and higher number of rings in NAs mixtures from OSPW were suspected to be more lipophilic. A low CMC is favoured by increasing the molecular mass of the lipophilic part of the molecule (Schramm et al., 2000). Rosen (2004) also states that increasing hydrophobicity of the surfactant, due to increases in chain length, decreases the CMC.

The higher hydrophobicity of the heavier molecular weight NAs present in OSPW, but less representative of commercial NAs, may facilitate micelle formation. The majority of the lower molecular weight NAs, present in OSPW and largely comprising commercial NAs mixtures, would remain as dissolved monomers and be readily biodegradable. Moreover, the high concentration of sodium in OSPW may serve to stabilize these negatively charged micelles. According to the electrostatic approach for surfactant micelle formation, the presence of monovalent counter ions such as Na^+ can act to stabilize the micellar state of anionic surfactants in aqueous environments (Tadros, 2000). Increased binding of counter ions due to increased electrolyte concentration also causes a decrease in the CMC (Rosen, 2004). The smaller size and lower molecular weight of commercial NAs may prevent the formation of stabilized micelles at a concentration range similar to the concentration of NAs present in OSPW (60 to 80 mg/L).

Thus, it is hypothesized that having a higher proportion of larger molecular weight congeners with greater degrees of cyclization, OSPW NAs mixtures form micelles at lower total concentrations than commercial NAs mixtures. This may result in greater NAs solubility and persistence in aqueous environments, which in turn may be correlated with the persistent chronic toxicity. The purpose of this investigation was to determine whether there are differences between commercial NAs and OSPW NAs in their ability to form micelles in aqueous solutions, and whether these differences, if present, are related to NAs mixture composition and salinity.

E.2. Materials and Methods

E.2.1 Test Solutions

Two test solutions were prepared using commercially available, Refined Merichem Naphthenic Acids (Merichem NAs) (Merichem Chemicals & Refinery Services LLC, Houston, TX). The first solution consisted of Merichem NAs dissolved in de-ionized Barnstead water. The second

test solution consisted of Merichem NAs dissolved in a stock salt solution with a total ionic content of 2342 mg/L, a level consistent with the level of salinity of OSPW. The concentrations of the individual salt ions were estimated to be 744 mg/L Na^+, 144 mg/L Cl^-, 1166 mg/L HCO_3^-, 224 mg/L SO_4^{2-}, 37 mg/L Ca^{2+}, and 27 mg/L Mg^{2+}. The standard, deoxycholic acid (Anatrace Inc., Cleveland, OH) was also tested, both on its own and dissolved in the stock salt solution. Deoxycholic acid is a commercially available anionic detergent standard with a CMC of 6 mM.

E.2.2 Critical Micelle Concentration Determination

The CMC of the two test solutions and two standard solutions were determined using the fluorescent probe 8-anilino-1-naphthalene sulfonic acid (Anatrace Inc.) (dye binding method) and a fluorescence spectrophotometer. Tests were conducted using excitation and emission wavelengths of 400 and 480 nm, respectively, with a gain setting of 2000. Incremental volumes of the test solutions (e.g., Merichem NAs) or standard solutions (e.g., deoxycholic acid) were added and the emission intensity at 480 nm measured. The fluorescence (emission intensity) was then plotted versus the total concentration of the NAs or deoxycholic acid (mM). The CMC is defined as the breakpoint in the fluorescence and can be obtained by visual inspection of the graph. Graphs were produced using Microsoft® Excel (2008 Version 12.3.0).

E.3 Results and Discussion

The CMC of deoxycholic acid was determined to be approximately 8 mM (Figure E.1). According to the technical specifications provided by Anatrace Inc., the CMC of deoxycholic acid is 6 mM. This slight deviation is likely the result of the small variation in the measurements, lack of replication, and the slightly subjective nature of determining the breakpoint visually. The visual estimation of the breakpoint concentration could have been improved by increasing the frequency of the readings around 6mM. Nevertheless, this method appears to be appropriate for determining the CMC. The CMC of Merichem NAs was estimated as 4 mM (Figure E.2), and it is believed that this is the first time that the CMC of Merichem NAs has ever been reported.

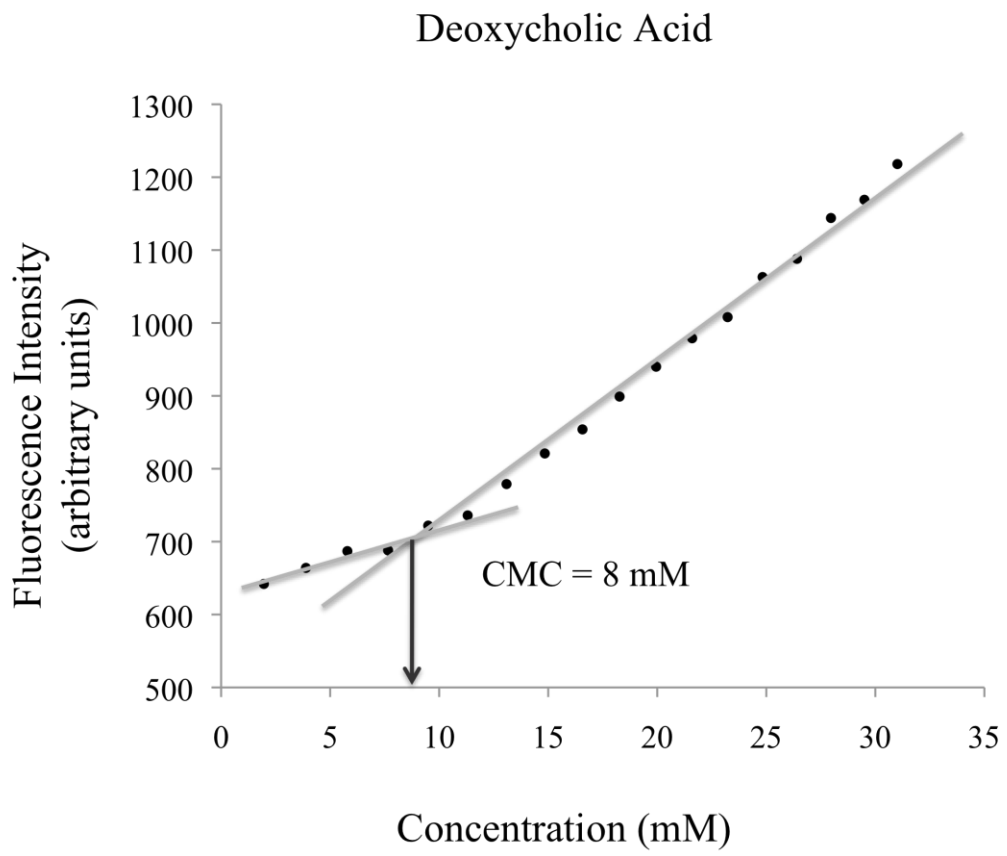


Figure E.1. Critical micelle concentration (CMC) of the standard compound, deoxycholic acid, as determined by the dye binding method using a fluorescence spectrophotometer.

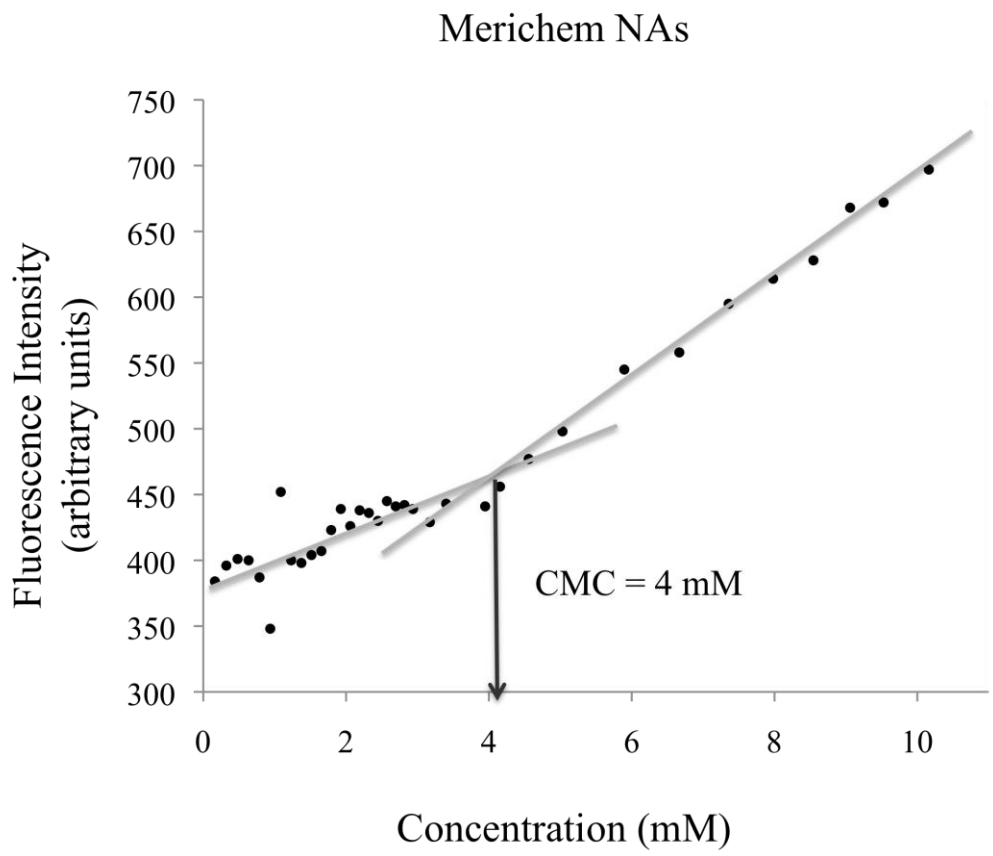


Figure E.2. Critical micelle concentration (CMC) of Merichem naphthenic acids as determined by the dye binding method using a fluorescence spectrophotometer.

The addition of salt to the test solution should result in a decrease in the CMC of both the standard and Merichem NAs (Schramm et al., 2000; Tadros, 2000; Rosen, 2004). The CMC of the standard deoxycholic acid decreased from 8 mM to 4 mM with the addition of dissolved salts (Figure E.3). The CMC of Merichem NAs also decreased from 4 mM to 3 mM (Figure E.4), however the effect was not as pronounced.

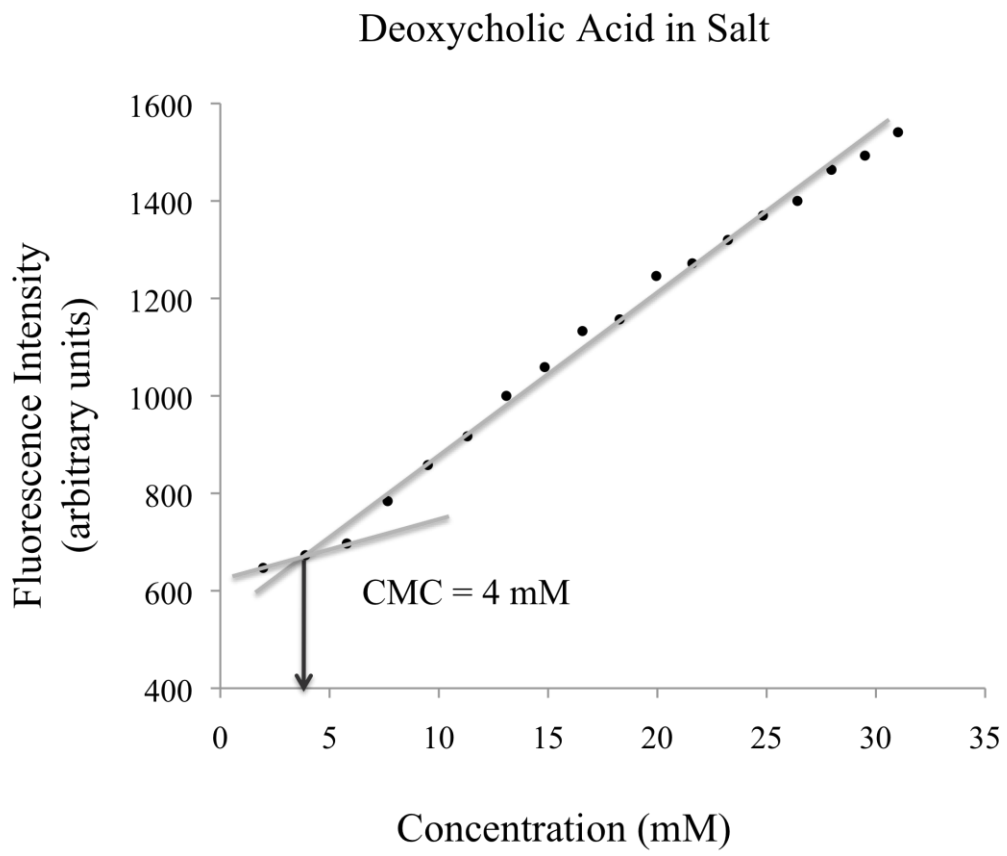


Figure E.3. Critical micelle concentration (CMC) of the standard compound, deoxycholic acid, in a salt solution (2342 mg/L total ionic content) as determined by the dye binding method using a fluorescence spectrophotometer.

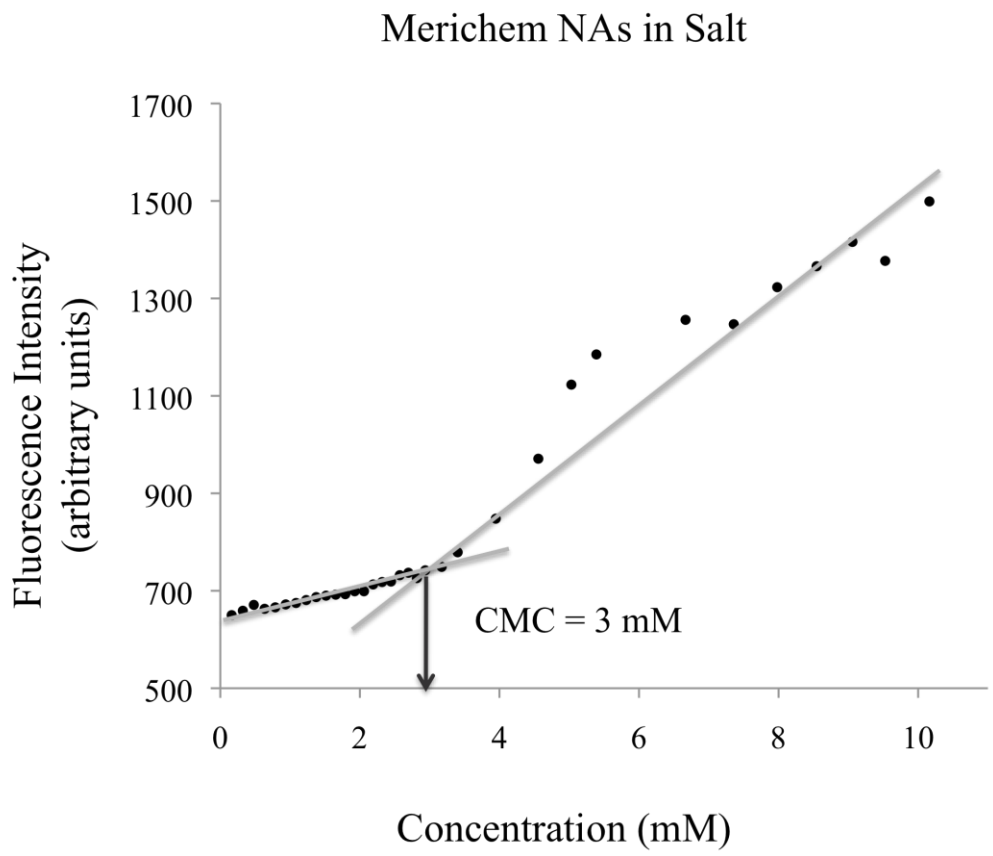


Figure E.4. Critical micelle concentration (CMC) of Merichem naphthenic acids in a salt solution (2342 mg/L total ionic content) as determined by the dye binding method using a fluorescence spectrophotometer.

C.5 Conclusions

It appears that that the hypothesis of micellar stabilization and decreased CMC due to increased electrolyte concentrations apply to Merichem NAs. The next step would be to repeat the experiment for extracted OSPW NAs, extracted OSPW NAs re-dissolved in a salt solution, and un-extracted NAs in un-manipulated OSPW from settling basins and detention ponds. It is hypothesized that the CMC would be lower for OSPW NAs, which contain higher molecular weight NAs, than the estimated CMC of the commercially available NAs used in this study. If an OSPW NAs mixture is ever successfully fractionated, it would be interesting to compare the CMCs of the different fractions in support of this hypothesis. Potential differences between the CMCs of the higher and lower molecular weight NAs might contribute to a more complete

understanding of NAs solubility, toxicity, sorption, selective biodegradation, and the readily degradable nature of commercial NAs compared to the more persistent OSPW NAs.

REFERENCES LIST FOR APPENDICES

Clemente, J.S., Yen, T.-W., Fedorak, P.M., 2003. Development of a high performance liquid chromatography method to monitor the biodegradation of naphthenic acids. J. Environ. Eng. Sci. 2, 177-186.

Frank, R.A., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van Der Kraak, G., Solomon, K.R., 2008. Toxicity assessment of collected fractions from an extracted naphthenic acid mixture. Chemosphere 72, 1309-1314.

Gaikar, V.G., Maiti, D., 1996. Adsorptive recovery of naphthenic acids using ion-exchange resins. React. Funct. Polym. 31, 155-164.

Headley, J.V., McMartin, D.W., 2004. A review of the occurrence and fate of naphthenic acids in aquatic environments. J. Environ. Sci. Health., Part A Toxic/Hazard. Subst. Environ. Eng. 39, 1989-2010.

Headley, J.V., Peru, K.M., McMartin, D.W., Winkler, M., 2002. Determination of dissolved naphthenic acids in natural waters by using negative-ion electrospray mass spectrometry. J. AOAC Int. 85, 182-187.

Herman, D.C., Fedorak, P.M., MacKinnon, M.D., Costerton, J.W., 1994. Biodegradation of naphthenic acids by microbial populations indigenous to oil sands tailings. Can. J. Microbiol. 40, 467-477.

Holowenko, F.M., MacKinnon, M.D., Fedorak, P.M., 2002. Characterization of naphthenic acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Res. 36, 2843-2855.

Leung, S.S., MacKinnon, M.D., Smith, R.E., 2001. Aquatic reclamation in the Athabasca, Canada, oil sands: naphthenate and salt effects on phytoplankton communities. Environ. Toxicol. Chem. 20, 1532-1543.

Lo, C.C., Brownlee, B.G., Bunce, N.J., 2006. Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids. Water Res. 40, 655-664.

Quagraine, E.K., Headley, J.V., Peterson, H.G., 2005. Is biodegradation of bitumen a source of recalcitrant naphthenic acid mixtures in oil sands tailing pond waters? J Environ. Sci. Health., Part A Toxic/Hazard. Subst. Environ. Eng. 40, 671-684.

Rosen, M.J., 2004. Frontmatter, in Surfactants and Interfacial Phenomena. Third Edition, John Wiley & Sons, Inc., Hoboken, NJ, USA.

Schramm, L.L., Stasiuk, E.N., MacKinnon, M.D., 2000. Surfactants in Athabasca oil sands slurry conditioning, flotation recovery, and tailings processes. In: Schramm, L.L. (Ed.). Surfactants: Fundamentals and applications in the petroleum industry. Cambridge University Press, Cambridge, UK, pp. 365-430.

Stasiuk, E.N., MacKinnon, M.D., 2000. Surfactants in Athabasca oil sands slurry conditioning, flotation recovery, and tailings processes. In: Schramm, L.L. (Ed.). Surfactants: Fundamentals and applications in the petroleum industry. Cambridge University Press, Cambridge, UK, pp. 365-430.

Scott, A.C., MacKinnon, M.D., Fedorak, P.M., 2005. Naphthenic acids in Athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. Environ. Sci. Technol. 39, 8388-8394.

Tadros, T., 2000. Surfactants. Kirk-Othmer Encyclopedia of Chemical Technology.